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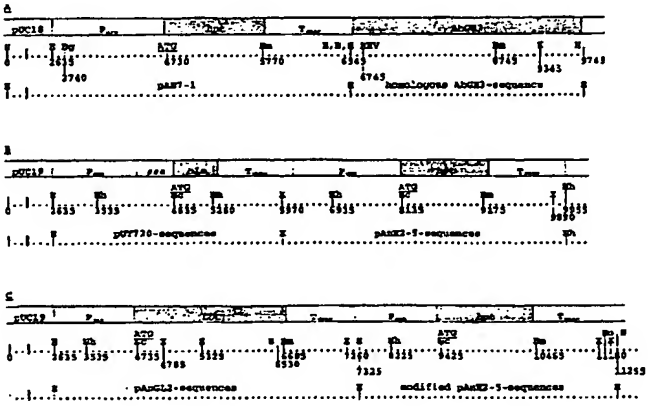
(54) Title: PRODUCTION AND APPLICATION OF TRANSGENIC MUSHROOM MYCELIUM AND FRUITBODIES



(57) Abstract

The invention involves different methods to modify genetic characteristics of homobasidiomycetes in particular commercial homobasidiomycetes such as the common or button mushroom *Agaricus bisporus* via treatment with donor DNA or fusions using protoplasts and via matings between strains. The methods may be used for the improvement of commercial characteristics and for the commercial production of enzymes and metabolites. The invention is in particular directed at a method for obtaining a selectable stable transformant of a homobasidiomycete capable of expressing integrated donor DNA comprising at least a dominant selectable marker at a detectable level, wherein said host is optionally non-auxotrophic and can be transformed without cotransformation with said dominant selectable marker and is transformed with said donor DNA. The invention is also directed at a method for production of stable transgenic fruitbodies directly from transformed heterokaryons or indirectly through mating or protoplast fusion of transformants obtained through mating of transformants obtained. A method for provinding a genetic fingerprint of both homokaryotic and heterokaryotic material obtained through transformation is also described as well as a method for producing homokaryotic, material from transformed heterokaryotic material. A specific vector for use in transformation is described as is a method for producing such a vector.

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Title: PRODUCTION AND APPLICATION OF TRANSGENIC MUSHROOM MYCELIUM AND FRUITBODIES.

FIELD OF THE INVENTION

The invention involves different methods to modify genetic characteristics of homobasidiomycetes in particular commercial homobasidiomycetes such as the common or button mushroom *Agaricus bisporus* via treatment with donor DNA or fusions using protoplasts and via matings between strains. The methods may be used for the improvement of commercial characteristics and for the commercial production of enzymes and metabolites.

BACKGROUND OF THE INVENTION

Fruitbodies from the common or button mushroom *A.bisporus* (Lange Imbach), also denoted *A.brunnescens* belonging to the class of basidiomycetes (order *Agaricales*), constitute an important crop in the Netherlands with a production of 190 million kilos in 1992. Almost 75% of this crop is exported. The United States of America, China and France are other important mushroom producing nations. General problems associated with the production and keeping of mushrooms involve e.g. infections by pathogens like *Pseudomonas tolaasii* or *P.agarici*, dsRNA viruses and browning caused by the action of endogenous poly-phenol-oxidases (PPO, like tyrosinase). To further improve product quality, conventional breeding programmes already carried out have been only moderately successful and may appear not to be sufficient on the long run, because this procedure is highly time consuming and because the genetic variation in commercially available strains is limited (Horgen et al. 1991).

The main problem for effective breeding strategies is caused by the rather abnormal life-cycle of *A.bisporus*, which involves the usual simultaneous segregation of either parental nucleus into one basidiospore. After outgrowth of this basidiospore heterokaryotic mycelium is formed containing nuclei and genetic characteristics that do not differ from those present in the parental mycelium. In addition, only little recombinational activity is observed during meiosis (Summerbell et al. 1989).

For this reason investigators all over the world have attempted for quite some years to develop a transformation system for commercial mushrooms such as *A.bisporus* for the introduction of novel characteristics. In other organisms, especially in plants the application of gene transfer technology is quite common and has already resulted in the first commer-

cial applications, but the absence of a transformation system generally applicable in a wild-type background in many basidiomycetes has strongly hampered molecular-biological research of such organisms, especially that of edible mushrooms.

Contrary to the situation in many ascomycetes, the application of antibiotic resistance markers for dominant selection of transformants of basidiomycetes has to date only been moderately successful. As a representative for heterobasidiomycetes, the phytopathogenic fungus *Ustilago maydis* has been reported to be transformable with both auxotrophic and antibiotic-resistance markers (Wang et al. 1988).

Within the class of homobasidiomycetes to which also edible mushrooms like *A.bisporus* and *Pleurotus ostreatus* (oyster mushroom) belong, *Schizophyllum commune* is considered a model organism to study genetics and developmental biology (Raper 1988). *S.commune* is in fact one of the first representatives of this class for which a transformation system was developed. This system is based on complementation of a *trp1* auxotroph with the homologous *TRP1* gene (Munoz-Rivas et al. 1986). However, it does not offer the possibility to transform non-auxotrophic homobasidiomycetes. So far, donor DNA comprising a prokaryotic dominant selection marker has not yet been found both integrated and expressed at a level which allows direct selection and stable maintenance of transformed homobasidiomycetes.

Successful transformations with auxotrophic markers have been described for *S.commune* (Munoz-Rivas et al. 1986), *Coprinus cinereus* (Binninger et al. 1987) and *Phanerochaete chrysosporium* (Alic et al 1989). In *P.chrysosporium* (Randall and Reddy 1992) and *P.ostreatus* (Peng et al. 1992) selection with antibiotic resistance markers was recently described, however the donor DNA sequences were not found to be integrated into the recipient genome and were subject to methylation. Peng et al describe the use of recombinant plasmid pAN7-1 for transformation with resistance to hygromycin B as selectable marker in *P.ostreatus*.

Similar processing of donor DNA was also observed in *S.commune* where the heterologous *E.coli hpt* (hygromycin B phosphotransferase) gene was introduced as antibiotic selectable marker by co-transformation in an auxotrophic strain of *S.commune* (Mooibroek et al. 1987). The heterologous sequences appeared to be heavily methylated, which may explain the low level of their expression (Mooibroek et al. 1990) and thus the difficulty to retrieve transformants by direct hygromycin B selection. It was

necessary to first select transformants for their ability to overcome the auxotrophic deficiency and subsequently to detect amongst those transformants a subset having hygromycin B resistance. The reverse selection, i.e. first for hygromycin B resistance did not produce results presumably due to low expression of the heterologous DNA and the consistent generation of false positive colonies that escaped the selective pressure but were not transformed. Similarly it was also suggested for *A.bisporus* that methylation of donor DNA or the low level of recombination in this organism might be causative for its resistance to genetic transformation (Royer and Horgen 1991). Despite numerous world-wide attempts, successful transformation of *A.bisporus* with heterologous DNA (Challen et al. 1991, Royer and Horgen 1991) leading to transformants comprising said DNA stably integrated and/or expressing it sufficiently to be detectable still has not been reported. The numerous unsuccessful attempts have been carried out with various strains (among them the *A.bisporus* strain U1) with the *hpt*-marker already mentioned as well as with other heterologous markers (Challen et al. 1991, Royer and Horgen 1991). It has been suggested that other markers should perhaps be included in the transformation experiments, e.g. ß-glucuronidase (GUS) gene for transient expression (Royer and Horgen 1991).

All data to date suggest that homobasidiomycetes are reluctant to genetic transformation with heterologous especially prokaryotic dominant selection markers. The results of attempting genetic manipulation of homobasidiomycetes include the perception that this reluctance may be due to insufficient expression and subsequently difficulties in detection of transformants.

One method currently used to obtain genetic variance in mushrooms is protoplast fusion. Teikoku-Pharm's Japanese patent e.g. discloses the preparation of a new strain of mushroom by protoplast fusion of *Lentinus edodes* and *P.ostreatus* exhibiting the taste of *L.edodes* and also exhibiting rapid growth. This method does not however lead to controlled manipulation of the characteristics of the resulting hybrid.

In Campbell Soup's US patent 4996390 commercial mushrooms of the genus *Agaricus* are disclosed containing genetic material from more than one species obtained by protoplast fusion. In particular the auxotrophic *A.bitorquis* (auxotrophic for nicotinic acid and resistant to cycloheximide) and a homokaryotic strain of *A.bisporus* (auxotrophic for adenine and uracil and resistant to cycloheximide) is described. This hybrid cannot form fruiting bodies, but can be crossed to form another

hybrid. Yet again this is not a method for selectively incorporating one or a few selected genes but is a method for random transfer of genetic material. Furthermore it suggests that auxotrophic species and species already comprising naturally occurring or mutagen induced resistance markers can only be used for efficient selection of hybrids.

DESCRIPTION OF THE INVENTION

Completely surprisingly during work on an *A.bisporus* strain 'Abade' to develop a transformation system based on complementation with the corresponding wild-type gene with a view to later applications in co-transformation systems along the lines of the previously mentioned approach for *S.commune* (Mooibroek et al. 1990) and *A.bisporus* transformation (Royer and Horgen 1991) a novel and efficient method of transformation of homobasidiomycetes was developed.

As the nature of the deficiency resulting in an adenine requirement and the mutation in the corresponding DNA sequences are unknown and in view of the large amount of work required to isolate the putative complementing sequence, transformation experiments were first initiated with available vectors containing the *E.coli hpt*-gene. The efficient transformation of this strain was completely unexpected in view of the fact that despite occasional satisfactory yields of protoplasts from *A.bisporus* U1 having been achieved, no transformants had previously been detected when using the same vector.

It was even more surprising to ascertain that successful use could be made of the heterologous *hpt*-marker for transformation of U1mp10, a new protoclone isolated from strain U1 via protoplasting and regeneration. This strain was selected for its similarity with 'Abade' with respect to early growth characteristics and colony morphology. Although U1mp10 yielded fewer protoplasts than 'Abade', strain U1mp10 also appeared to be transformable with the *hpt*-gene, contrary to strain U1 or other protoclones thusfar. These data indicate that the development of the transformation systems now available for 'Abade' and U1mp10 could not be anticipated on the basis of current knowledge of *A.bisporus* transformation.

The subject invention is directed at a method for obtaining a selectable stable transformant of a homobasidiomycete capable of expressing integrated donor DNA comprising at least a dominant selectable marker at a detectable level, wherein said host is optionally non-auxotrophic and can be transformed without cotransformation with said

dominant selectable marker and said host is transformed with said donor DNA. The method according to the invention can successfully be used on commercial strains of homobasidiomycetes, such as strains belonging to the genus *Agaricus*. It has thus become possible to produce transgenic mycelium and fruitbodies by the introduction of foreign and/or homologous DNA sequences and the expression thereof resulting in new genetic and/or phenotypic characteristics in said mushroom mycelium and/or fruitbodies. The transgenic mushroom material may also be applied for the transfer of transformed nuclei to mushroom mycelium of the same strain, another strain of the same species, another species of the same family or another species of another family. The transgenic organism or any part thereof may further be used for the production of foreign and/or homologous proteins, (poly)peptides and/or metabolites. The proteins, (poly)peptides and/or metabolites may be recovered from the organism's tissues and/or from the medium. The method may be used for the production of strains with improved quality aspects, such as reduced levels of browning (e.g. PPO-activity), for the production of pathogen resistant strains and for the genetic marking of commercially interesting strains of mushrooms to establish proprietary rights. It has now become possible to integrate specific desired heterologous nucleic acid sequences in a homobasidiomycete and maintain said nucleic acid sequence in the transformed mycelium and the fruiting body resulting therefrom, without the need for sustained selective pressure.

For the subject specification a dominant selectable marker is meant to be a marker that is selectable in a wild type of the host to be transformed i.e. a host without auxotrophic deficiencies. It is therefore now possible to transform homobasidiomycetes strains that are not auxotrophic with a selectable marker.

The method of transformation according to the invention in the embodiments just described can in particular be very successfully carried out on a host obtained by subjecting homobasidiomycete material to a transformation procedure, wherein the host to be transformed exhibits delayed differentiation in comparison either to non-protocloned homobasidiomycete material and/or the wild type strain U1 as obtainable from ATCC, said delayed differentiation being macroscopically visible in the form of amended morphology due to a change in the number and/or height of aerial hyphae, preferably by the absence of aerial hyphae and/or by a diminished hyphae aggregate formation preferably by the

absence of hyphae aggregates. In particular a method wherein the host belongs to the strain 'Abade' is an embodiment that provides good results. 'Abade' exhibits delayed differentation when compared to U1 as obtained from ATCC. A strain exhibiting amended morphology of the required type for use in a method of transformation according to the invention can be suitably obtained by subjecting homobasidiomycete material to protocloning followed by selection of resulting homobasidiomycete material exhibiting the desired delayed differentiation. A suitable host can also be obtained by subjecting homobasidiomycete material to a rejuvenation procedure followed by selection of resulting homobasidiomycete material exhibiting the desired delayed differentiation. Preferably the transformation procedure commencing with the protoplast formation is carried out as soon as possible after detection of the occurrence of delayed differentiation. With a view to preventing loss of the transformability of the rejuvenated homobasidiomycete material it is preferable to commence the transformation procedure with the formation of protoplasts using material which has not been subjected to more than 25 outgrowth phases. An outgrowth phase in this respect comprises selecting a colony, plating out the colony and growing the colony on a 9 cm agar plate until the plate is full and a person skilled in the art will recognise that equivalents to the number and length of outgrowth phases thus defined also fall within the scope of the invention. Extremely good results were achieved when less than five outgrowth phases took place between selection of the desired rejuvenated host homobasidiomycete material and protoplast formation as part of the transformation procedure. It is supposed that the transformability can be lost if the number of outgrowth phases is too high.

The good results achieved are in particular illustrated by the results of transformation of the strains 'Abade' and U1mp10 with donor DNA comprising a dominant selectable marker in a manner according to the invention. In the description of the experiments a detailed method of transformation of these strains with plasmid DNA comprising the *hpt* gene as dominant selectable marker is given. To our knowledge no investigations were conducted or attempts undertaken for the description of the 'Abade' flat or thin type of morphology. The morphology of 'Abade' colonies was never observed in any other commercial or non-commercial strain of *A.bisporus*, except shortly after protoplasting and regeneration on CMPS-medium. On different media tested, including MMP, CMP, DT80, MS

and B5, it has a brownish to light-brown, translucent appearance, lacking any macroscopically visible aerial hyphae emerging from the main colony which is attached to the agar medium, nor does it show any aggregation of hyphae. The different morphology was apparent directly after purchase from the ATCC strain collection. The 'Abade' morphology shows resemblance with the phenotypes described e.g. for *S.commune thn* (thin) mutants (Wessels et al., 1991a). In the *thn* mutant described the expression of the *Sc*3-hydrophobin gene is blocked as well as the generation of aerial hyphae. It has been suggested that hydrophobins accumulate in the cell walls of the hyphae that excrete them and contribute to the formation of hydrophobic crosslinked structures within the cell wall. The *S.commune thn* mutation is analogous to the *Streptomyces bld* mutation. In *A.nidulans*, disruption of the analogous *rodA* gene encoding a hydrophobin-like protein resulted in a decreased hydrophobicity of aerial mycelium (Stringer et al., 1991, for review see Chater, 1991). In addition to the formation of aerial hyphae, hydrophobin genes are also involved in fruitbody formation in *S. commune* (Wessels et al., 1991b). The possible reduced levels or even absence of these or other similar crosslinked structures in the cell walls of "Abade' might contribute to better digestibility of the cell walls by lytic enzymes resulting in higher yields of protoplasts observed by us. It is difficult to imagine that the known adenine-auxotrophic mutation in 'Abade' would have such a dramatic effect on cell wall synthesis. Therefore, we assume that other defects may be present in 'Abade'. However, we cannot rule out the possibility that some key functions would be defective resulting in pleiotropic effects on both adenine-requirement and cell wall assembly. It may be significant to note that the *ade* mutation in 'Abade' does not result in absolute adenine-auxotrophy. After transfer of mycelial inocula on media without adenine and longterm incubation, we have observed some slow growth, perhaps feeding on the inner parts of the colony. This phenomenon of slow growth has been associated by others with instability of the *ade* mutation.

In addition, the known adenine-requirement of 'Abade' cannot solely explain its better transformability.

Strain U1mp10 was isolated (with other similar colonies) after protoplasting and regeneration on CMPS-medium of strain U1, which was also purchased from the ATCC-collection (ATCC62462). In addition to a number of normal appearing regenerates, the U1mp10-type of colonies were isolated because of their resemblance with the 'Abade'-phenotype. The

frequency of their generation was about 0.08. This phenotype was still retained after the regeneration plate was fully covered with outgrown regenerates. At the moment of the isolation of inocula for further propagation, the colonies touched neighbouring colonies (mp stands for mating protoclones). In addition to the flatter type of growth the U1mp10-type of colony also revealed densely packed hyphae, especially in the middle of the colonies. Another form of altered morphology was apparent i.e. the marked reduction of the presence of hyphal aggregates as is visible by absence of a thread-like morphology. After further propagation it appeared that the aberrant morphology was transiently retained on CMP and MMP agar medium, with a later tendency to form aerial hyphae. For the production of cultures on MMP + cellophane and subsequent cultivation in liquid medium the outer differentiating parts of the colonies were avoided. From the later experiments, including α-exterase isozyme-analysis and fruitbody induction from U1mp10-derived transformants, it was concluded that the U1mp10-type of colony was not a homokaryon, but a special type of heterokaryon. The primary U1mp10-transformants either had the U1 phenotype (50%) or the U1mp10 phenotype (50%). After prolonged cultivation all primary transformants formed sectors with the U1 phenotype.

It is well-known that protoclones derived from a heterokaryotic parental strain may demonstrate a tremendous variation in morphologies. In plant biotechnology this phenomenon is denoted somaclonal variation. These findings are best explained with the assumption that imbalances have occurred during protoplast formation and/or regeneration with respect to the presence of cellular organelles and/or nuclei. These phenomena may well represent a stress to the organism which is accompanied by the development of competence for transformation. Numerous examples exist of improving transformation efficiencies by pretreatment of the host or the donor DNA with (UV)-radiation or carcinogens. This was in fact the reason why we attempted the use of linear DNA's which provides numerous double-strand breaks. It is well-known from other systems that these double-strand breaks may induce repair mechanisms and recombination machineries. It is anticipated that in "Abade', which only yields one type of regenerates, this stress may be constantly present if not only the ade mutation is present, but also other undefined mutations.

The efficiency of protoplast formation cannot be the only reason for transformability of *A.bisporus*, because from U1mp10 successful transformation experiments were conducted with 2.7×10^6 protoplasts.

Therefore, we assume that some elements involved in the morphological aberrations, possibly by the action of pleiotropic effects, may improve the competence for genetic transformation, e.g. by the induction of S.O.S.-systems.

In a further preferred embodiment of the invention the donor DNA is linearized prior to transformation as this leads to a more positive effect on the transformation efficiency. This positive effect of the use of DNA that is linearized prior to transformation on transformation efficiency is in line with observations in other organisms than homobasidiomycetes such as the yeast *Hansenula polymorpha* (Faber et al. 1992) and some filamentous fungi (Banks et al. 1992, Liou et al. 1992, Tsai et al. 1992).

Any commonly acceptable method for transformation of protoplasts can be used in the method according to the invention, such as electroporation, use of PEG or particle bombardment. A person skilled in the art will be able to determine which method best suits the homobasidiomycete material to be transformed, whether it has been derived by protoplasting or not. The use of electroporation to mediate uptake of donor DNA is a good choice due to the cell type specific controllability of parameters. Furthermore the use of electroporation eliminates the risk of aggregate formation of protoplasts by PEG and thus prevents potential segregational instability of the donor DNA. This effect may be even more realistic for multinucleate *A.bisporus*.

In another preferred embodiment of the method according to the invention the efficiency of transformation can be significantly increased by taking measures to ensure a higher yield in the number of protoplasts per unit time prior to the actual transformation step. Methods are known to a person skilled in the art for increasing protoplast yield and regeneration efficiency (Sonnenberg et al 1988). It is pointed out here that the known methods for increasing protoplast formation as such are insufficient to obtain transformability of the host homobasidiomycete material. This was illustrated by the non transformability of U1. As however the chances of finding transformants are increased by such measures the inclusion of such measures in the method according to the invention is preferred.

As disclosed the rejuvenation is an important aspect of the subject method. Rejuvenation as such is a known procedure (Fritsche 1991) and can occur in the form of natural rejuvenation or artificial rejuvenation. Natural rejuvenation occurs in the form of mating homokaryotic spore

cultures leading to mating products which are considered to be rejuvenated offspring. In the method according to the invention further homokaryotes can be derived from the rejuvenated offspring for transformation. The artificial method of rejuvenation comprises formation of protoplasts, regeneration and selection of homokaryons therefrom.

In particular the invention is thus directed at a method for obtaining a dominant selectable stable transformant of a homobasidiomycete capable of expressing stably integrated donor DNA comprising at least a dominant selectable marker at a detectable level, said method comprising

a) subjecting the mycelium of the host to be transformed to at least:
   1) protoplast formation, followed by
   2) an outgrowth phase to colonies, followed by
   3) isolation of individual protoclones resulting from step 2, followed by
   4) an outgrowth phase to colonies followed by
   5) a selection of a protoclone resulting from step 4 on the basis of exhibiting a delayed differentiation in comparison either to non-protocloned homobasidiomycete material and/or the wild type strain U1 as obtainable from ATCC, said delayed differentiation being macroscopically visible in the form of amended morphology due to a change in the number and/or height of aerial hyphae, preferably by the absence of aerial hyphae and/or by a diminished hyphae aggregate formation preferably by the absence of hyphae aggregates.
   6) optionally at least one cycle of further propagation of a selected clone, including a subsequent outgrowth phase to colonies and cultivation, preferably in liquid medium
   7) at least one protoplast formation step from such a colony

and subsequently

b) subjecting protoplasts resulting from step 7 to transformation with donor DNA. With the method according to the invention the host to be transformed can optionally be non-auxotrophic and can also be transformed with said dominant selectable marker without cotransformation.

The indicated pretreatment of protocloning the host material to be transformed prior to the actual transformation step appears to improve the success rate of transformations. Parallel transformation experiments

in which U1 and U1mp10 were compared yielded only transformants from U1mp10, but not from U1 indicating the preferred use of protocloning.

In a more preferred embodiment of this method according to the invention the material to be used in step b) for transformation has not been subjected to more than 5 successive outgrowth phases in step 6. The use of recently protocloning material for the transformation apparently enhances the success rate of obtaining transformants from a commercial strain, which was previously not transformable.

In a further preferred embodiment of the subject method of protocloning and transformation of such protoplasts with donor DNA comprising a dominant selectable marker the protoplast formation step can be further improved by growth of fungal mycelium in plant medium for cultivation and regeneration of plant cells e.g. MS medium (Murashige and Skoog 1962) immediately preceding step 7. This additional measure has been found to be particularly effective for a host belonging to the genus *Agaricus*. It seems that the application of MS-medium during cultivation in liquid medium causes a less densely packed type of mycelium and perhaps also prevents the deposition of extracellular or cell wall specific metabolites that might interfere with the activity of the lytic enzyme used for protoplasting. On the other hand MS-medium lacks some essential components (which are present in DT80) needed for long term cultivation of *Agaricus*. The shift from a rich medium to a poorer medium for the induction of competence for genetic transformation is also well known for other transformation systems e.g. *Bacillus subtilis* and *E.coli*.

For the transformation methods according to the invention in the various embodiments described above the transformation can be successfully carried out using donor DNA comprising at least a dominant selectable marker. The selectable marker can for example encode resistance to an antibiotic and/or a fungicide. The resistance encoding sequence can suitably encode for resistance against hygromycin B. Such resistance can be provided by the *hpt* gene. The gene sequence is known for hpt from both *E.coli* and *Streptomyces*. The sequence from *E.coli* is used in the Examples and is eminently suitable for use in a method according to the invention.

The donor DNA can further comprise at least one nucleotide sequence homologous to a part of the DNA of the non-transformed host. The presence of such a homologous sequence can imply the presence of a sequence at which integration is desirable in the host chromosome, i.e. in order for site specific homologous recombination to occur. It can also imply the

presence of homologous control sequences in order to ensure optimal processing of the donor DNA by the host. Preferably the donor DNA further comprises a promoter and optionally a terminator sequence homologous to the host to be transformed.

In general no critical demands other than the usual ones on shuttle vector composition generally applicable and well known to a person skilled in the art of transformation of other organisms, in particular basidiomycetes beside the already mentioned presence of the dominant selectable marker are made on the vector to be used.

It has however been found that a specific new vector can quite successfully be used for transformation purposes of the *hpt* gene as dominant selectable marker. The vector is obtainable by at least the following essential steps:

1) Introduction of an *Nco*I-site comprising the methionine-encoding translation initiation codon of the *hpt* gene e.g. via the PCR method using the wild-type *E.coli hpt*-gene or plasmids pHRC or pAN7-1 as templates and combinations of the following primers having seq. id 1, 2 as illustrated in the sequence listing.
2) Removal of the unique *Nco*I- and *Eco*RI-sites from the wild-type *hpt* coding region by in vitro mutagenesis e.g. via PCR using primers having sequence id 3 and 4.
3) Cloning of the fragment altered in steps 1 and 2 in a proper *E.coli* (e.g. pUC-based) vector.
4) Introduction of *Eco*RI/*Nco*I genomic fragments preferably from *A.bisporus* and optionally comprising promoter activity.
5) Introduction of *Bam*HI-*Hin*dIII genomic fragments preferably from *A.bisporus* and optionally comprising terminator activity.
6) Propagation of this plasmid DNA in a proper host strain preferably lacking or mutated for the capacity to restrict and/or modify cloned homobasidiomycete DNA.

The invention covers this vector and also use thereof in any of the transformation methods according to the invention. In particular a vector according to the invention comprises a promoter controlling the dominant selectable marker of the donor DNA, said promotor being derived from the host to be transformed. For transformation of *A.bisporus* a vector comprising an *A.bisporus* promoter is for example preferred. Suitably a pAN7-1 vector can be modified when e.g. an *A.bisporus* promoter replaces the *A.nidulans* promoter. Preferably a strong promoter sequence will be used to control expression of the dominant selectable marker in a vector

according to the invention. The strength of the promoter can generally be derived from the level of expression of a gene so that any promoter of a gene encoding a product that is present in large amounts will be suitable. An eminently suitable promoter for transformation vectors for *A.bisporus* is the *GPD-2* promoter sequence. A pAN7-1 modified vector comprising the *A.bisporus GPD-2* promoter sequence instead of the *A.nidulans* promoter sequence controlling expression of the *hpt*-gene is comprised within the invention.

Also termination sequences that can be recognised both by *A.nidulans* and *A.bisporus* are preferably absent in a vector according to the invention. In the case of the pAN7-1 vector being modified for use in *A.bisporus* as a vector according to the invention the termination sequences such as present in the *TRPC* terminator sequence of *A.nidulans* are preferably removed.

A further improvement of a vector according to the invention, in particular a pAN7-1 modified vector comprises the presence of a mutated *E.coli hpt* gene as gene encoding the dominant selectable marker. The mutated *E.coli hpt* gene comprises a CG duplet at position 799 from the 5'-*Nco*I-site comprising the ATG start codon instead of the native GC duplet. Consequently the mutant *hpt*-gene encodes Ile-Val instead of Met-Leu. The mutated sequence driven by proper expression signal sequences resulted in a higher resistance level of *E.coli* transformants. A vector comprising any other mutation in a *hpt* gene resulting in a higher resistance level of host transformants is included in the scope of the invention.

For homologous integration it is desirable to include a sequence of nucleic acid homologous to a part of the chromosome of the host to be transformed. A suitable sequence for *A.bisporus* comprises the AbGH3 sequence, a sequence that can be isolated through hybridisation with putative *N crassa* tyrosinase sequences obtained by PCR with degenerate primers and published sequences (Lerch 1992) in a manner known to a person skilled in the art. Any number of alternatives will be apparent to a person with knowledge of homologous integration and with access to the known DNA sequences of the host to be transformed.

A preferred vector according to the invention for transforming *A.bisporus* will comprise the homologous strong promoter *GPD-2* controlling the dominant selectable marker, the *E.coli hpt*-gene with the mutated duplet disclosed above and will also comprise a sequence of homologous

nucleic acid for *A.bisporus* such as AbGH3 if homologous integration is desired.

It is possible to also carry out cotransformation with the transformation method in the various embodiments of the invention disclosed. Besides the transformation with the donor DNA comprising the dominant selectable marker as primary marker as described it is possible to introduce by co-transformation further DNA comprising a desirable sequence to additionally transform the host. The technique of co-transformation allows the introduction and stable integration of any DNA sequence together with the primary (e.g. *hpt*) selectable marker. In order to increase co-transformation efficiencies, in particular in *A.bisporus* which exhibits low (±10%) cotransformation efficiencies, transformation vectors can be constructed comprising both the primary selectable marker and the cotransforming sequence (figure 19). The resulting transformants comprising the cotransformed DNA can be demonstrated by Southern blot analysis. The cotransforming DNA may code for any homologous or heterologous polypeptide or protein which may or may not be excreted, thus affecting metabolic and biochemical potential of the transformant when expressed in the proper tissue and at the proper growth stage. Specific homologous or heterologous genes may also be over-expressed by using a strong (e.g. *GPD*-2) promoter or by insertion of higher copynumbers of the same gene. GPD which is a constitutive enzyme, may constitute about 5% of total cellular protein. The addition of rDNA sequences might also favour the integration of multiple copies. Alternatively, a specific gene may be repressed by different techniques. In the yeast *Saccharomyces cerevisiae* and some fungi, like *A.nidulans* and *Neurospora crassa*, where homologous integration may occur relatively frequently, gene-disruption may be the best technique to silence a specific gene (Fincham 1989). The probability of the occurrence of homologous (site-specific) integration has been correlated with the length of the homologous insert of the donor DNA plasmid with linearization of the donor DNA plasmid within the homologous insert sequence. We have applied this system by cloning the AbGH3-*Hind*III-fragment into the unique *Hind*III site of pAN7-1 generating pHAG3-1, followed by digestion of the unique *Kpn*I-site within the AbGH3-sequence. Phenomena like antisense RNA inhibition and co-suppression are common in plant genetic engineering, but may not be applicable in heterokaryotic fungal transformants containing only one (co)transformed nucleus, unless both nuclei have been transformed directly or combined by mating or protoplast fusion. Co-transformation with sequences coding for

specific antibodies (in plants denoted 'plantibodies' Hiatt 1990), however, may find general applicability. It appeared that also parts of antibodies comprising the variant (Fv) chains and expressed in *E.coli* may have sufficient binding capacities (Plückthun 1990), possibly also for application in fungi (fungibodies).

Desirable sequences for example suitable for cotransformation using the transformation procedure according to the invention are the putative *A.bisporus* tyrosinase genes (isolated at our institute) cloned sense or antisense, the *A.bisporus* mannitol-dehydrogenase gene or the glucose-6-phosphate dehydrogenase gene (Wood et al. 1991), the *A.bisporus* methallothionein genes (Nishiyama et al. 1990), e.g. the barley α-thionin gene (Gausing 1987) or resistance to dsRNA viruses through cross-protection using a gene coding for e.g. a coat protein (Harmsen et al. 1989, Harmsen et al. 1991). In particular these sequences can be introduced into *A.bisporus* protoplasts by cotransformation according to the invention. It is in fact possible to insert multiple nucleic acid sequences from the cotransforming vectors at the same site in the chromosome. This is probably due to in vivo ligation of nucleic acid from the various vectors after linearization of the vectors has occurred such that compatible sticky ends or blunt ends are created prior to the integration event. Compatible sticky ends can be created simply e.g. by digestion of the vectors with the same restriction enzyme(s). It is also possible to insert sequences in tandem using one vector.

With the above mentioned methods for transformation that have now become available it is possible to produce stable transgenic fruitbodies directly from transgenic heterokaryons, like U1mp10 primary transformants, or by matings or protoplast fusions between two compatible strains wherein at least one of the mating strains is a transformant obtainable from such a transformation method according to the invention. For example a suitable transformant to be used for such a method is an 'Abade' transformant comprising resistance to hygromycin B as transgenic selectable marker and comprising an adenine deficiency as does the non-transformed strain 'Abade'. This transformant can advantageously be mated with another mating strain that is not deficient for adenine and is also sensitive to hygromycin B, resulting in selectability of the product of said mating on both lack of adenine deficiency and resistance to hygromycin B. Such mating can take place by generally known techniques such as naturally occurring anastomosis or artificial protoplast fusion.

It is also possible to produce homokaryotic material from transformed heterokaryotic material obtainable through the transformation method according to the invention. These homokaryons can be used for matings. As already indicated a method of providing a genetic fingerprint specific for a transformed homobasidiomycete comprising DNA analysis of a transformant or a transgenic fruitbody obtainable through a transformation method according to the invention also falls within the scope of the invention. In particular such a method is described, wherein a genetic fingerprint specific for heterokaryotic material resulting from a method of mating using a transformant obtainable through the transformation method according to the invention as at least one of the mating strains, can be determined distinguishing said heterokaryotic material from the homokaryotic transformant used as mating strain by analysing for the presence of more or different genetic material in the heterokaryotic material than in the homokaryotic transformant. This can be carried out for example by counting the number of nuclei per cell or using protoplasts analysed in a cell sorter on the basis of the presence of about twice the amount of genetic material in the heterokaryotic mating product than in the homokaryotic transformant. Even more elegantly, use can be made e.g. of 'Abade' transformant C25-1 which contains the donor DNA integrated at the homologous AbGH3-sequence. Mating products with this strain as one of the two mating partners contain the native 3.5 kb AbGH3-*Cla*I-fragment and the C25-1/*Cla*I fragment which has a much higher molecular weight (depending on the number of plasmid copies integrated). When the second mating strain comprises different genetic material than the transformant mating strain the genetic fingerprint can be further completed with an analysis of the RFLP, RAPD or isozyme band pattern of the resulting heterokaryotic material and comparison thereof to the starting material or any other known strains can be used to ascertain proprietary rights.

The invention is also directed at non-auxotrophic transgenic homobasidiomycete material derived from a non-auxotrophic homobasidiomycete, said transgenic material comprising stably integrated donor DNA comprising a dominant selectable marker such as a resistance to antibiotic and said transgenic material further being capable of expressing said donor DNA in an amount sufficient to ensure selectability over the corresponding non transgenic material.

Certain aspects of the invention just described are further elucidated in the following detailed disclosure of the invention.

DETAILED DISCLOSURE OF THE INVENTION

GENERAL METHODS

1- Source and growth of mycelia

Strains from *A.bisporus* were purchased from the American Type Culture Collection (ATCC 24663, denoted 'Abade', and ATCC 62462, commercial strain U1), inoculated on MMP agar medium containing Malt Extract (1%, Oxoid), Mycological Peptone (0.5%, Oxoid) and agar (1.5%) and propagated at 24°C for 1-2 weeks for 3 generations each. Thus, the availability of inoculation material of identical generations was guaranteed for all individual experiments. Except mycelia needed directly for further processing, stocks were kept at 8°C. In order to prepare liquid cultures for the production of protoplasts, MMP plates containing a cellophane sheet were loaded with 5-10 inocula each and grown for 5-10 days at 24°C. Colonies were subsequently scraped off the cellophane and macerated for 20 seconds in a Waring Blender, containing 50 mL of MSG20 (Murashige and Skoog 1962, containing 20 $g.L^{-1}$ glucose) medium. For 'Abade' 20 $\mu g.mL^{-1}$ adenine was added. The amount of macerated mycelium that corresponded with the material derived from two plates was inoculated in each Fernbach flask containing a final volume of 150 mL. Depending on the strain used growth was allowed while standing for 3-7 days at 24°C. Protoplasts were usually isolated from the mycelium grown in 3-4 Fernbach flasks.

2- Preparation and regeneration of protoplasts

The Fernbach cultures were rinsed thoroughly over cheese-cloth with sterile milliQ water and finally with 0.6 M sucrose. The mycelium was then transferred to an Erlenmeyer flask containing 0.6 M sucrose and 10 $mg.mL^{-1}$ Novozym 234 (Sigma or Interspex Products Inc.) and incubated for 2-3 hours at 24°C. The formation of protoplasts was monitored microscopically with regular intervals. Their number amounted usually 10^8-10^9 protoplasts per experiment for 'Abade' and 10^6-10^7 for strains U1 and U1mp10. Protoplasts were purified by sequential filtration through cheese-cloth and 50 mL-syringes containing about 2 g of glass-wool, previously rinsed extensively with 0.6 M sucrose and pelleted for 30 min at 3000 rpm at 8°C using a Heraeus Christ centrifuge accommodating 4-6 100 mL tubes. Pellets were further purified by 2 washes with 0.6 M sucrose and 1 wash with SEH-electroporation buffer containing 0.6 M sucrose, 1 mM EDTA, 1 mM HEPES, pH 7.0, each time by centrifugation in

mL Corex tubes for 5 min at 3500 rpm at 4°C using a Beckman RC-5C centrifuge and the HB4 swinging bucket rotor. Protoplasts were finally resuspended in 100 µL ice-cold SEH-buffer per parallel (usually 3-4) experiment. Alternatively, protoplasts can also be produced by growing colonies on membranes (e.g. Gene Screen, Dupont de Nemours and Co. Inc. NEN Products) layered on top of agar media containing a cellophane membrane. After growth, the membrane is incubated upside-down in the protoplasting solution for 1-2 h at 24°C. Protoplasts can be recovered from the liquid phase and washed as described above. Regeneration of protoplasts was accomplished in two ways: 1- by plating directly onto CMPS agar medium (for the isolation of protoclones) or 2- by incubating in liquid CMPS for 3-5 days (for transformation experiments, see 4-transformation procedure).

3- Cloning procedures, preparation of plasmid DNA and Southern blot analysis of transformants

Cloning procedures were carried out essentially according to Maniatis et al. (1982). Transforming plasmid DNA was isolated using CsCl density gradient centrifugation or according to the Qiagen maxiprep extraction protocol. Transforming plasmids were pAN7-1 (Punt et al. 1987) and pHAG3-1 (a derivative of pAN7-1 containing a 3 kb random *A.bisporus* genomic *Hin*dIII-fragment (which weakly hybridised to a *N.crassa* laccase specific oligonucleotide [Lerch 1982] cloned in the unique *Hin*dIII restriction site of pAN7-1). Plasmid pAN7-1 was linearized with *Hin*dIII, pHAG3-1 with *Kpn*I, which is located in the *A.bisporus* insert sequence, thus yielding two *A.bisporus* DNA termini. Restriction enzymes were used according to the suppliers recommendations (Pharmacia). The DNA was then dialyzed for 30 min on Millipore VM membranes floating in a solution containing 10% glycerol and 1mM EDTA or the DNA was phenol/chloroform-purified.

For the identification of transformants Southern blot analysis was performed on genomic DNA isolated essentially according to Raeder and Broda (1985), and comprising restriction enzyme digestions using conditions recommended by the supplier (Pharmacia, with 10-fold excess of restriction enzymes), electrophoresis, blotting and digoxigenin-dUTP/AMPPD (DIG) autoluminescence detection (Boehringer Mannheim).

A new set of multipurpose fungal transformation vectors has also been constructed, which allow the convenient exchange of *Eco*RI-*Nco*I promoter fragments, the exchange of *Nco*I-*Bam*HI structural gene fragments

and/or the exchange of *Bam*HI-*Hin*dIII terminator sequences. The construction of these novel vectors is described in Example 2.

4- Transformation procedure

Isolated protoplasts were resuspended in ice-cold SEH-electroporation buffer and immediately electroporated with about 10 µg of donor DNA using the BioRad Gene Pulser (parameters, electrode gap: 0.2 cm; voltage: 0.45 kV; capacity: 25µF; shunt resistance: 200 Ω), mixed immediately with 10 mL of CMPS medium (Compost extract 25% v/v, Mycological peptone 0.5% w/v, plus sucrose, 0.6 M) containing 100 $\mu g.mL^{-1}$ cefotaxim (Duchefa) and incubated for 3-5 days at 24°C to regenerate cell walls. Then the suspension of regenerates was warmed briefly at 38°C and mixed with 1 volume of 2% SeaPlaque low melting point agarose plus sucrose 0.6M (38°C) and poured as overlays (5 mL per plate) on DT80 (Dijkstra Tween 80) medium (Dijkstra 1976, Sonnenberg et al. 1988) containing 10, 25 or 50 $\mu g.mL^{-1}$ hygromycin B (Duchefa) plus adenine (20 $\mu g.mL^{-1}$) for 'Abade', or on DT80 or B5 (plus glucose, 2%) medium containing 50 or 100 $\mu g.ml^{-1}$ hygromycin B for U1mp10. Viability was tested before and after pulse delivery with serial dilutions on CMPS agar medium. Plates were incubated at 24°C. Regenerates became visible by microscopy after about 3 days. Transformants arose macroscopically after 1 to several weeks.

5- Cotransformation

As an example of a method of cotransformation according to the invention co-transformation with pUT720, comprising the phleomycin (*ble*)-resistance gene from *Streptoalloteichus hindustanus* is described here. In addition, experiments with the *Photinus pyralis* (firefly) luciferase gene as reporter system were carried out.

Cotransformations were performed after linearizing both plasmids (e.g. pAN7-1 and pUT720 by digestion with *Hin*dIII) after mixing equal amounts of DNA containing the primary selectable marker and the cotransforming DNA (10-25 µg). Hereby, the native and/or modified ble and/or mutated *LUC* genes (see below) were used as reporter genes for the determination of expression levels after integration into the recipient genome.

The *hpt* gene constructs described in example 2 have been used for promoter probing and promoter trapping (promoter fishing) experiments in a manner known per se to find promoters that are activated at specific

growth stages or under specific conditions. For this purpose *A.nidulans* promoter sequences were removed by digestion followed by electrophoresis and isolation of the proper vector-containing band from the gel by the Qiaex extraction protocol. For promotor-probing experiments random genomic *A.bisporus* fragments were ligated into the promoterless vector for transformation of *E.coli*. From all *E.coli* transformants obtained the plasmid DNA (now containing a variety of genomic *A.bisporus* fragments) was re-extracted and used for *A.bisporus* transformation as linear molecules. In case *LUC* or *ble* genes were used as reporter genes, the constructs were introduced with the *hpt* gene as primary selectable marker, by co-transformation. The promoter sequences may be recovered by techniques like marker or plasmid rescue, inverse PCR or PCR with one specific anchor-primer annealing to the donor DNA plus a random (RAPD) primer. For plasmid or marker rescue the genomic DNA of transformants is digested with a restriction enzyme, which does not cut the donor DNA introduced. Several restriction enzymes may be applicable separately. The total genomic DNA is then ligated to make the donor DNA plus the flanking genomic sequences circular, which can then be used to transform appropriate *E.coli* strains. The *E.coli* strain may be propagated to isolate the new recombinant plasmid for further analysis, including retransformation of *A.bisporus*. In case of tandem integrations the PCR-mediated techniques may be more suitable.

6- Marking of transformants

Southern blot analysis of 'Abade' and U1mp10 transformants obtained according to the invention revealed specific banding patterns upon restriction of the total DNA with *Bgl*II, *Bam*HI or *Eco*RI (or their combination), which cut the original vectors and upon hybridization with the *hpt*-probe. For each transformant analyzed the unique position of either single or multiple integrations was determined by the flanking genomic *Bgl*II, *Bam*HI or *Eco*RI restriction sites. Thus for any transformant the number of hybridizing fragments generated and their specific sizes after single or multiple digestions is unique and may therefore be regarded as a transformant-specific and thus strain-specific fingerprint. In addition, the unique flanking DNA sequences for each individual transformant can be determined following inverse PCR and sequencing.

7- Stability of transformants

The mitotic stability of 'Abade' transformants was assessed in two ways. Primary hygromycin B resistant colonies were grown on non-selective MMP agar medium covered with a sheet of cellophane for several (10) 'generations', each time by transferring inocula from the edge of the colony. Phenotypic expression of the *hpt*-gene was checked for growth on DT80 medium containing 10 or 25 $\mu g.mL^{-1}$ hygromycin B and 20 $\mu g.mL^{-1}$ adenine. The non-transformed strain and a colony that grew on a selective plate without having received any donor DNA (false positive) were taken as controls. It may be significant to note that with the experimental protocols now developed no or hardly any false positive colonies appear. U1mp10 transformants demonstrated a sectored type of growth resembling the parental U1 strain. Sectors were investigated separately for retention of the *hpt*-gene and the hygromycin B resistant phenotype in the same way as for the 'Abade' transformants.

The stability of transformants was also investigated by Southern blot analysis after growth of selected colonies on MMP agar medium. The position of autoluminescent signals detected after hybridization with the *hpt*-probe were compared with the positions of ethidium bromide-fluorescent bands from the corresponding gel. This procedure shows whether the donor DNA has been integrated into the recipient genome or is present within the cells as free plasmids. By selecting proper restriction enzymes conclusions can also be drawn with respect to the integrated nature of the donor DNA.

8- Production of homokaryotic transformants from heterokaryotic primary transformants

Primary U1mp10 hygromycin B resistant transformants were inoculated with up to five inocula per plate on GeneScreen (Dupont de Nemours and Co. Inc, NEN Products) hybridization membranes layered onto MMP agar medium and grown at 24°C for about 7 days. Membranes containing the colonies were then transferred upside down to petridishes containing 10 mL of 0.6 M sucrose containing Novozyme 234 (10 $mg.mL^{-1}$). After incubation for 2-3 hours at 24°C protoplasts produced were recovered from the protoplasting solution by centrifugation for 10 min at 3500 rpm and washed three times with 0.6 M sucrose by repeated centrifugations. Protoplasts were incubated for 3 days at 24°C in petridishes containing 10 mL of liquid CMPS medium to regenerate cell walls. Serial dilutions were then plated onto DT80 agar medium containing 10, 25 or 50 $\mu g.mL^{-1}$ hygromycin B. Similar procedures have been described earlier for the production of

homokaryotic breeding material from heterokaryotic parental strains. Homokaryons were generated with an efficiency of approximately 10% (Sonnenberg et al. 1988). The nuclear constitutions were assessed e.g. using α-esterase isozyme analysis (Sonnenberg et al. 1988) and/or RAPD-mapping (Khush et al. 1991).

9- Mating of transformants

For the production of transgenic fruitbodies from *A.bisporus*, a heterokaryotic constitution of the mycelium is required, which should contain at least two compatible nuclei with opposite mating types. Unless the initial transformant is already heterokaryotic (e.g. from U1mp10), heterokaryosis between homokaryotic strains or even between a homokaryotic and a heterokaryotic strain (socalled Buller phenomenon, Raper et al. 1972) may be accomplished by naturally occurring anastomosis, or artificially by protoplast fusions (see below).

For the initiation of anastomosis, inocula of about 2-5 mm^2 were placed upside down onto non-selective MMP agar medium, about 1-5 mm apart. Inoculated plates were incubated at 24°C for 1-4 weeks. Within the zone where outgrowing colonies touch, compatible interactions may be observed resulting in pigmentation, in an aberrant mycelial morphology and the frequent excretion of intracellular material resulting in the formation of brown droplets in e.g. *P.ostreatus* (Kay and Vilgalys 1992). Similar phenomena were also observed for matings described here. Inocula from the interaction zones that also included some parental mycelium were transferred to new agar media for further analyses described below in example 5.

When a homokaryotic transformant, which is marked via the presence of a known Southern blot banding pattern produced after digestion of the genomic DNA with said or other restriction enzymes followed by Southern blot analysis with appropriate probes (described in section 'Marking of strains'), is mated with another preferably homokaryotic strain with a different RFLP-, RAPD- and/or isozyme banding pattern, the resulting heterokaryotic strain yields the same banding pattern as the original transformed 'Abade' homokaryon used as mating strain. This method is referred to as indirect marking of strains. The parental homokaryotic transformed strain can be distinguished from the new heterokaryon by additional counting of the number of nuclei per cell, by cell sorting of protoplasts or by RFLP-, RAPD-techniques and/or by isozyme analyses. An elegant example is given by using 'Abade' C25-1 as one of the mating

partners. In true heterokaryons the presence of two different AbGH3-fragments can be demonstrated, due to the modified size of the AbGH3-ClaI fragment through homologous integration of pHAG3-1.

10- Stability of mated transformants

Heterokaryotic transformants that were generated by matings between 'Abade' hygromycin B resistant transformants and U1 protoclones, were transferred to non-selective MMP-medium covered with a sheet of cellophane and propagated for several 'generations', each time by taking one inoculum from the edge of a colony of about 5 cm in diameter to new MMP-plates plus cellophane, and another inoculum to test the ability to grow on double selective SD-medium containing hygromycin B (50 $\mu g.mL^{-1}$). After further propagation the remaining colony was removed from the cellophane and subjected to Southern blot analysis to verify the presence of the donor DNA.

11- Protoplast fusions with transformed protoplasts

Hygromycin B or phleomycin resistant transformants from *A.bisporus* can be used for the production of intra- or interspecies hybrids by protoplast fusions followed by dominant selection of fusion products.

Transformants, preferably (made) homokaryotic, derived e.g. from U1mp10 and expressing the *hpt* or *ble* gene, may be fused to one another and fusion products may be dominantly selected by the simultaneous application of hygromycin B and phleomycin contained in the growth medium. They may also be fused to other organisms, containing another endogenous or donated dominant selection marker.

'Abade' transformants expressing the *hpt* or *ble* gene may be used for the dominant selection of fusion products with protoplasts expressing the complementing *ADE* gene, from any wild-type organism, but preferably from *A.bisporus*, *A.bitorquis*, *A.arvensis* or other *Agaricus spp.* Fusions of protoplasts have already been accomplished between *A.bisporus* and *A.bitorquis* (Patent number US4996390; 91-302364/41), between *P.ostreatus* and *Lentinus edodes* (Patent number JP4173034; 92-255378/31) and between a variety of different fungal species (Patent number J02245179; 90-339235/45). However, none of these fusions relied upon the presence and dominant selection of a donor DNA marker. Similarly, other fungal protoplasts e.g. from *P.ostreatus* or *L.edodes* or even from plants e.g. *Solanum tuberosum*, which are sensitive to a hygromycin B concentration of about 50 $\mu g.mL^{-1}$ or a phleomycin concentration of about 7.5 $\mu g.mL^{-1}$ may be used

for the dominant selection of hybrids. In each case the transformed 'Abade' transformant donates the antibiotic resistance, whereas the fused counterpart donates the functional, complementing *ADE* gene.

Protoplasts from *Agaricus spp* (or e.g. *P.ostreatus*) were prepared as described above for 'Abade' and *A.arvensis* or according to Sonnenberg et al. (1988) and finally resuspended in 0.6 M sucrose. Then about equal amounts (100 µL, containing 10^7-10^8 protoplasts) of either protoplast type were mixed in a 15 mL pointed centrifuge tube (Corex). One volume of 40% PEG6000 was added by pipetting carefully onto the centrifuge tube wall. By this way the PEG solution formed the lower layer at the interphase, covered by the protoplast suspension. Protoplasts were then centrifuged for 5 min at 800 rpm in a swinging bucket rotor using a table centrifuge (Beckman) onto the interphase, which allowed the formation of fusions. After standing for 5 min at room temperature 5 mL of 0.6 M sucrose were added and carefully mixed by inversion. Subsequently, the protoplast suspension was washed three times by centrifugation for 5 min at 2500 rpm in a table centrifuge and resuspended in CMPS for 3 days to regenerate cell walls and plated onto DT80 agar medium containing the proper antibiotic. Serial dilutions were plated onto CMP agar medium to determine the efficiency of regeneration.

12- Production of transgenic fruitbodies

MMP agar plates covered with cellophane were inoculated with 5 inocula each of the desired transformed, mated or fused strain. Plates were incubated at 24°C for 10-15 days. Liquid cultures were prepared in Fernbach flasks containing 150 mL MSG20 (supplemented with 100 $\mu g.mL^{-1}$ Cefotaxim) and the macerated material of 2 plates for each flask. The Fernbach flasks were incubated at 24°C for 3-5 days. Then for spawning 100 grams of wheat-grains were sterilized in the presence of 50 ml of demineralized water by autoclaving for 20 min. The sterilized wheat grains were added to the liquid Fernbach cultures and incubated at 24°C for about 2-3 weeks, dependent on the strain used. The total contents of overgrown wheat-grains plus remaining MSG20 medium were mixed with about 750 grams of pasteurized ready to use compost (CNC, Milsbeek, The Netherlands) and incubated in a 5-10 L polystyrene box. The box was incubated closed, at 24°C for 2-3 weeks. Subsequently, a layer of casing soil of about 3 cm (CNC, Milsbeek, The Netherlands) was added on top of the compost now overgrown by the mycelium. The box was incubated closed at 24°C for another 5-8 days and then transferred to 16°C and a relative

humidity of 75-90%. The lid was then placed on 4 wooden pins in the corners of the box leaving a ventilation gap of 2-3 cm. The surface of the casing soil was moistened daily. After several days fruitbodies start to emerge.

Fruitbodies can also be produced in jars essentially as described but with adapted amounts of mycelial inocula, grain kernels, compost and casing soil. Details are described in the legends to figure 17.

Example 1: Protoplasting and regeneration of 'Abade' and *A.arvensis*

Mycelia of 'Abade' and *A.arvensis* were grown in principle as described earlier (Sonnenberg et al. 1988) except for a growth phase in liquid MSG20-medium (Murashige and Skoog 1962, containing 20 $g.L^{-1}$ of glucose as a carbohydrate source) instead of DT80. With this medium we obtained extremely high yields of 'Abade' protoplasts of over $5.7x10^8$ per 4 Fernbach flask cultures. Under similar conditions from *P.ostreatus* $1.1x10^9$ protoplasts were produced, suggesting the general applicability of this medium for the production of protoplasts from edible fungi.

Example 2: Construction of multipurpose transformation vectors

A novel convenient set of multipurpose transformation vectors has been constructed based on pUT720 purchased from Cayla, Toulouse, which contains pUC19 vector sequences, the *Aspergillus nidulans GPDA* promoter plus the *Trichoderma reesei* cellobiohydrolase I (*SSA*) excretion signal sequences, contained in a 2.2 kb *Eco*RI-*Nco*I fragment, the *Streptoalloteichus hindustanus* phleomycin-resistance (*ble*) gene cloned as a 0.44 kb *Nco*I-*Bam*HI fragment and the *A.nidulans* 0.76 kb *Bam*HI-*Hin*dIII *TRPC*-terminator fragment. After removal of the *A.nidulans GPDA Eco*RI-*Nco*I promoter fragment this vector can be used for the insertion of *Eco*RI-*Nco*I promoter fragments from any organism, but preferably from *A.bisporus*. Before or after exchange of the promoter fragments, the *Nco*I-*Bam*HI *ble* gene fragment may be replaced by any structural gene, but preferably by the modified *E.coli* 1.0 kb *hpt Nco*I-*Bam*HI fragment or the *Photinus pyralis* (firefly) 1.9 kb *LUC* (luciferase)-gene also contained in an adapted *Nco*I-*Bam*HI fragment. The modifications are described below. The latter *LUC*-containing construct may be used for transient expression assays, preferably in fungal protoplasts. Finally, the *A.nidulans TRPC* terminator sequence may be exchanged by any, but preferably *A.bisporus* terminator sequence, contained in a *Bam*HI-*Hin*dIII fragment. The vectors thus constructed may be

used for the isolation and *in vivo* selection of random genomic sequences with promoter or terminator activity from any organism, but preferably from *A.bisporus*. This technique is called promoter or terminator fishing, respectively.

Modified *hpt* construct.

The *E.coli hpt* 1.0 kb *Bam*HI fragment (Gritz and Davies 1983), cloned into pAN7-1 was used to introduce a 5'-*Nco*I site encompassing the ATG translational initiation codon by PCR overhang-extension. The *hpt* gene and 5'-adjoining sequences of plasmid pAN7-1, which is able to transform a number of different fungi (Punt et al. 1987) and which also was used for transformation of the basidiomycetes *Schizophyllum commune* (Mooibroek et al. 1990) and *Pleurotus ostreatus* (Peng et al. 1992), was used as a template for PCR reactions. Figure 3 shows the schematic reconstruction of the *hpt* gene, containing a 5'-*Nco*I site, and with deleted *Nco*I and *Eco*RI-sites within the coding region. The PCR primers used for different steps in vector constructions are shown in table 1.

Primer Nco-HPT5 introduces an *Nco*I-site encompassing the translation initiation codon (ATG) of the *hpt* gene. Furthermore, introduction of this *Nco*I-site changes the second codon of the *hpt* gene in pAN7-1 from proline to alanine. AnTRPC3C primes at the 3' end of the *TRPC* terminator of pAN7-1. The PCR reaction yielded a 1.7 kb *hpt-TRPC* terminator fragment, which was isolated and digested with *Bam*HI, thereby separating the *hpt* gene from the *TRPC* terminator. Subsequently, partial digestion with *Nco*I was performed and a 1.0 kb fragment corresponding to the *hpt* gene was isolated and ligated into *Nco*I/*Bam*HI-digested vector pMTL23, yielding plasmid pMHN.

In order to remove the internal *Eco*RI- and *Nco*I-sites of the *hpt* gene in this construct (located at positions 244 and 352 relative to the ATG), two phosphorylated primers were designed which mutate the *Eco*RI- and *Nco*I-sites without changing the amino acid sequence (primers HPT-E2 and HPT-N2C, respectively). With these primers a 0.1 kb subfragment of the *hpt* gene was synthesized on template pAN7-1. Plasmid pMHN was digested with *Eco*RI, followed by partial digestion with *Nco*I at position 352, and treatment with Mungbean nuclease and CIP (calf-intestine alkaline phosphatase) to remove protruding ends and 5' phosphate groups. This vector was used to ligate the 0.1 kb PCR fragment, resulting in plasmid pMHMut carrying the *hpt* gene as a *Nco*I/*Bam*HI fragment in pMTL23. Sequencing of the new hpt gene revealed a change in the nucleotides at

position 799 from the ATG start codon (see below). This change improved the resistance level of recombinant *E. coli* and the transformation efficiency of *A.niger*. The *hpt* gene from pMHMut was cloned as an *Nco*I/*Bam*HI fragment into vectors with different promoters and the *A.nidulans TRPC* terminator (see below).

Table 1: PCR primers used for vector constructions

Code	Sequence
Nco-HPT5	Sequence id 1 5' GAC ATC ACC ATG GCT GAA CTC 3' ATG, translation initiation site of *hpt* gene
AnTRPC3C	Sequence id 2 5' CCG CTC GAG TGG AGA TGT GGA 3' complement
HPT-E2	Sequence id 3 5' pAG TTC AGC GAG AGC CTG ACC 3' p, primer phosphorylated; A, position 244 relative to ATG of *hpt* gene
HPT-N2C	Sequence id 4 5' pCAT AGC CTC CGC GAC CGG CT 3' complement; p, primer phosphorylated; C, position 356 relative to ATG of *hpt* gene
EN-LUC-1	Sequence id 5 5' GGG AAT TCC ATG CC ATG GAA GAC GCC AAA AAC ATA 3' ATG, translation initiation site of *LUC* gene
T7	Sequence id 6 5' TAA TAC GAC TCA CTA TAG GG 3' complement

AbGPD1 Sequence id 7
5' G GAA TTC GTT GTC ATC ACC GCT CCT GGG AG 3'
G, position -1074 relative to ATG of *GPD*^{Ag2} gene

AbGPD3 Sequence id 8
5' GAA GAA GAA TTC AGA GGT CCG CAA GT 3'
G, position -290 relative to ATG of *GPD*^{Ag2} gene

AbGPD2c Sequence id 9
5' GCT TAT CGC CAT GGT TTG TCT CTC 3'
complement; CAT, complement of ATG of *GPD*^{Ag2} gene

PR-HPT1 Sequence id 10
5'-ATG.AAA.AAG.CCT.GAA.CTC.ACC.GCG.ACG.TCT-3'
(encompassing ATG at position 1)

PR-HPT2c Sequence id 11
3'-GGG.TCG.TGA.GCA.GGC.TCC.CGT.TTC.CTT.ATC-5'
(complement, encompassing TAG around position 1050)

PR-LUC-1 Sequence id 12
5'-ATG.GAA.GAC.GCC.AAA.AAC.ATA.AAG.AAA.GGC-3'
(encompassing ATG at position 304)

PR-LUC-2c Sequence id 13
3'-CAC.CGG.GGC.CGA.CTT.AAC.CTT.AGC.TAT.AAC-5'
(complement, encompassing position 1896)

Modified luciferase construct.

In order to introduce an *Nco*I-site encompassing the ATG of the *LUC* gene from *P.pyralis*, primer EN-LUC-1 was designed. This primer was used together with the (standard) T7 primer and plasmid pT3T7-LUC (Promega) in a PCR reaction which yielded a 1.8 kb *LUC* fragment. After digestion with *Nco*I and *Bam*HI the *LUC* gene was ligated into vector pMTL23 digested with *Nco*I and *Bam*HI, which yielded plasmid pLUC-N. The *LUC* gene was cloned as an *Nco*I/*Bam*HI fragment into vectors with different promoters and the *A.nidulans TRPC* terminator (see below). The integrity of the mutated *hpt*- and *LUC*-genes was assessed by positive expression in *E.coli* after cloning

in appropriate expression vectors. The modified hpt gene also resulted in a higher transformation efficiency in *A.niger*.

Promoter constructs.

In *A.bisporus* two *GPD* genes have been detected which are separated by a 0.29 kb intergenic region. Only the downstream GPD^{Ag2} gene is active in mycelium (Harmsen et al. 1991). The promoter region of this gene is used in new transformation vectors because of its high level of expression and because it normalizes the generation of mRNA when fused to the hpt gene (see below). Two different sized promoter fragments were isolated via PCR. A 0.29 kb fragment, representing the intergenic region of the two *GPD* genes, was isolated using primers AbGPD3 and AbGPD2c. Primers AbGPD1 and AbGPD2c were employed to obtain a 1.0 kb fragment which extends into the coding region of the upstream GPD^{Ag1} gene. Both fragments have the *Nco*I-site, encompassing the translation initiation site of the GDP^{Ag2} gene, as the 3' end. The two fragments were isolated by PCR on genomic DNA from both *A.bisporus* U1 and 'Abade'.

Primers AbGPD1 and AbGPD3 introduced an *Eco*RI-site at the 5' end of the PCR fragments. Thus, the fragments were cloned as *Eco*RI/*Nco*I fragments into *Eco*RI/*Nco*I-digested pUT720, thereby replacing the *A.nidulans GPD* promoter by the *A.bisporus GPD* promoter fragments. This procedure yielded plasmids pFAAG1 and pFUG1 (containing 1.0 kb *GPD* promoter fragments from 'Abade' and U1, respectively) and pFAAG2 and pFUG2 (containing 0.29 kb *GPD* promoter fragments from 'Abade' and U1, respectively). These plasmids therefore, contain *GPD* promoter sequences fused to the *S.hindustanus ble* gene and the *A.nidulans TRPC* terminator.

These plasmids were digested with *Nco*I and *Bam*HI to replace the *ble* gene by the *LUC* gene which was isolated after *Nco*I/*Bam*HI digestion of plasmid pLUC-N (see 'Modified luciferase construct'). The resulting constructs were named pAbAGL1 and pAbUGL1 (containing 1.0 kb *GPD* promoter fragments from 'Abade' and U1, respectively) and pAbAGL2 and pAbUGL2 (containing 0.29 kb *GPD* promoter fragments from 'Abade' and U1, respectively).

Furthermore, the *LUC* gene was ligated directly into *Nco*I/*Bam*HI-digested pUT720, creating construct pAnGL1, and into pUT720, in which the *Eco*RI/*Nco*I *A.nidulans GPD* promoter fragment including the signal sequence from the *SSA* cellobiohydrolase I gene from *T.reeset* was replaced by the *Eco*RI/*Nco*I *A.nidulans GPD* promoter fragment from pAN8-1, yielding pAnGL2.

Analogous to the construction of the *LUC*-containing plasmids described above, constructs were made with the modified *hpt* gene from plasmid pMHMut. These constructs are: pA1H (analogous to pAbAGL1), pU1H (analogous to pAbUGL1), pA2H (analogous to pAbAGL2), pU2H (analogous to pAbUGL2), pAnH1-5 (analogous to pAnGL1) and pAnH2-5 (analogous to pAnGL2).

The methods described here may be applied for the construction of transformation vectors with any, but preferably *A.bisporus* promoter sequence and/or with any, but preferably *A.bisporus* transit signal sequence and/or with any homologous or heterologous structural gene or fusions thereof and/or with any, but preferably *A.bisporus* terminator sequence.

Example 3: Transformation of 'Abade' protoplasts through electroporation with linear plasmid DNA.

Figure 1 shows hybridization signals from linearized plasmids pAN7-1 (6.5kb) and pHAG3-1 (9.5kb), lanes 1 and 2, respectively, from genomic 'Abade' DNA, lane 3 and from the genomic DNA isolated from colonies all recovered from selective plates (DT80 plus hygromycin B and adenine). Strains A10-1 and A25-1 had not been exposed to any donor DNA, thus representing the well known false positives which were only detected during early transformation attempts. Strains B10-1 and B25-2 had originally been exposed to native (non-digested) pAN7-1. Strain B25-2, lane 7, is apparently also a false positive, whereas B10-1 is a true transformant characterized by the *hpt*-specific hybridizing signal at the position of the non-digested chromosomal DNA, indicating that the donor DNA had been integrated into the 'Abade' genome. Integration was also observed after electroporation with linear pAN7-1 (C25-1 and C25-2, lanes 8 and 9, respectively) and with linear pHAG3-1 (D10-1, D10-2, D10-3 and D25-1, lanes 10, 11, 12 and 13, respectively). No free plasmid migrating at the approximate position of the linear plasmids has been observed at any occasion. These data also support the stable nature of the hygromycin B resistant transformants.

Figure 2 shows a Southern blot analysis of the genomic DNAs from four 'Abade' hygromycin B transformants after digestion with different restriction enzymes. In each transformant bands were detectable at the same position as the linearized pAN7-1 or pHAG3-1 plasmids, indicating that within the transformants tandem repeats had been formed during processing of the donor DNA. In addition, bands migrating at other

positions are detectable as well (except in lanes 5 and 6, from transformant C25-2), indicating that double- or multiple integration events had occurred, which is quite common in fungal transformants. In addition, bordering fragments from the recipient also contribute to the generation of new bands. Figure 2 also shows that the individual transformants can be distinguished on the basis of their unique banding patterns, which may be considered a strain specific fingerprint.

The same procedure here described for transformations with the *hpt* gene was also successfully applied with the phleomycin resistance (*ble*) gene present in pUT720 and pAN8-1. These vectors contain the *A.nidulans GPD* promoter region, the *S.hindustanus ble* gene and the *A.nidulans TRPC* terminator region. Plasmid pUT720 also contains *T.reesei* cellobiohydrolase I (*SSA*) excretion signal sequence (see example 2). The donor DNA was linearized via *Hin*dIII digestion. After electroporation and regeneration (as described for the *hpt* gene), regenerates were plated onto DT80 plates containing 5 or 7.5 $\mu g.mL^{-1}$ phleomycin (Cayla, Toulouse).

Example 4: Transformation of U1mp10, a derivative of commercial strain U1.

Protoplasts from strain U1mp10, which was isolated as described were transformed by electroporation with *Hin*dIII linearized pAN7-1. The total number of protoplasts used for each individual experiment amounted $2.7x10^6$. A number of $2.3x10^4$ regenerates was counted after plating serial dilutions and incubation at 24°C for about 7 days. From a DT80 plate containing 25 $\mu g.mL^{-1}$ hygromycin B one transformant was obtained and from plant B5 medium (Duchefa, Netherlands) containing 50 or 100 $\mu g.mL^{-1}$ hygromycin B in total 7 transformants were recovered within 3 weeks without any background growth. Figures 4 A, B and C show Southern blot analyses of all U1mp10 transformants isolated in this experiment. The same insertional characteristics were observed as for 'Abade' transformants described above, which is support for the stability of integrated donor DNA (figure 4A).

The primary transformants had either the U1-phenotype (50%) or the U1mp10-phenotype (50%), immediately after isolation from the selective plate and further propagation on MMP. One major difference observed in U1mp10 and 'Abade' hygromycin B resistant transformants was the consistent occurrence of differentiating sectors in those derived from U1mp10 upon transfer to non-selective MMP-medium. In order to investigate the segregational stability of the donor DNA, inocula from individual

sectors were transferred to MMP-medium plus cellophane. Different sectors had a different growth rate. Between 1-4 weeks after inoculation the material was freeze-dried and subjected to Southern blot analysis. Figure 4B shows that with one exception in sector 3 from transformant U1mp10/Bb100-2 all sectors had retained the donor DNA. In the same transformant sector 1 and 2 had slightly different banding patterns. Figure 4C shows that transformant specific fingerprints were generated which allows discrimination between one another but also between 'Abade' transformants (figure 2).

Example 5: Production and dominant selection of *A.bisporus* heterokaryons containing an *ade/hyg*^{R} and a wild-type nucleus

New strains were produced after mating homokaryotic hygromycin B-resistant transformants from 'Abade' and preferably homokaryotic protoclones from commercial strain U1 as described in the General procedures section 'Mating of transformants'. Figure 5 shows a representative result of experiments in which inocula from said interaction zones and both parental mycelia were grown on double selective SD-medium (Yeast Nitrogen Base without amino acids, prepared according to the suppliers instructions with glucose as a carbohydrate source, Difco Laboratories, USA) thus lacking adenin and supplemented with hygromycin B (50 $\mu g.mL^{-1}$). Only inocula from said interaction zones exhibited growth contrary to either parental strain. When fresh matings were attempted between either parental strain directly onto said double selective agar medium, no growth was observed, indicating that the mating procedure described was essential to yield *ADE/hyg*^{R} colonies.

To rule out the possibility that double selected colonies were just mixtures of parental mycelia, protoplasts were produced, regenerated in liquid CMPS-medium and plated onto double selective SD-medium. The results demonstrate that with a high efficiency of about 90% again *ADE/hyg*^{R} colonies were formed. In about 10% of the regenerates no growth was observed by microscopy. These results confirm the heterokaryotic status of double selected crosses.

Example 6: Analysis of transformant DNA using PCR.

Total DNAs were extracted according to the protocol described earlier. Samples (100 ng) of template DNAs were subjected to PCR analysis with primers indicated in the legend to figure 6. PCR was performed using *Taq*-polymerase in 30 cycles of 1 min 94°C, 1 min 55°C and 1 min 72°C. PCR-

products were electrophoresed in 1.0% agarose in TAE (Tris-HCl, acetate, EDTA)-buffer stained with ethidium-bromide.

Figure 6 shows clear banding patterns of specific PCR-fragments comprising parts of the tandem *GPD1* and *GPD2* genes of *A.bisporus* (lanes 2, 3, 4 and 5), indicating that both Abade and U1 have the same, or at least compatible, template DNA sequence of *GPD*-genes, which is support for the proper classification of Abade as an *A.bisporus* species, despite its aberrant colony morphology. From two putative (pAN7-1)-transformants in lanes 7 and 8, PCR-fragments with the same size were generated as in lane 9, which contained plasmid pAN7-1 as a template, contrary to the non-transformed Abade control in lane 6. The results show that (stably or transiently) transformed Abade strains (C25-1 and D10-1) can be obtained using the *hpt*-gene and procedures described and that the transformed status can be assessed using PCR and the primers listed above.

Conclusions regarding the integrated nature of donor DNA sequences is shown by Southern blot analysis in a number of other examples.

Example 7: Identification of transformed fruitbodies derived from U1mp10.

Fruitbodies were produced as described in the general methods. The Southern blot analysis shown in figure 7 clearly demonstrates the presence of donor DNA sequences in each fruitbody sample analyzed except for the primary transformant (U1mp10/Bb100-2) that had lost the donor DNA in one of the three sectors observed (figure 4B). This result indicates that the donor DNA has been stably maintained throughout fruitbody development, despite the absence of any selective pressure in favour of the *hpt*-containing nuclei. There is no sign of differential segregation of any one of the transformed nuclei. In at least three transformants identical hybridization patterns were obtained in the starting mycelium and the derived fruitbodies. In cases of doubt, additional digestions with other restriction enzymes and/or the determination of adjacent genomic sequences may provide conclusive discrimination. It can be concluded that donor DNA sequences are transmitted to *A.bisporus* fruitbodies with high frequency and usually with hybridization patterns identical to the mycelial pattern. These patterns can suitably be used to assess proprietary rights.

Example 8: Southern blot of transformant DNA derived from 'Abade'.

Total DNA was isolated and Southern blot analysis was carried out as described in the general methods section.

All mating products shown in figure 8 contain the two bands which are also visible in the transformed Abade parents (D10-1 and C25-1, lanes 1 and 2) and the pAN7-1 control (lane 19). This indicates the presence of pAN7-1 sequences in the genome of the mating products. As expected, the parental U1 protoclones do not contain any sequences hybridizing to the *hpt*-probe. The faint signal present in lane 12, panel C, is most likely due to overflow of DNA from the neighbouring slots. The hybridizing bands remain present for three generations in all mating products. However, additional bands appear in later generations. Whether these are caused by partial digestion or by rearrangements of the transforming DNA, or other donor DNA processing events, remains to be investigated. It can therefore be concluded that the mating products between Abade transformants and U1 protoclones stably maintain pAN7-1 sequences in the genome for at least three generations.

Example 9: Cotransformation with pAN7-1 and pUT720.

Before transformation both plasmids pAN7-1 and pUT720 were linearized with *Hin*dIII. After electroporation of Abade protoplasts as described in the general methods section, transformants were selected on hygromycin-containing medium. DNA from the hygromycin-resistant colony D20-1 was isolated and subjected to Southern blot analysis as described in the general methods section.

The restriction enzymes used for Southern blot analysis shown in figure 9, all have only one recognition site in both pAN7-1 and pUT720. Thus, the bands of approximately 6 kb in panel A corresponding to the size of plasmid pAN7-1 indicate the presence of tandemly integrated copies of pAN7-1 in the genome. *Nco*I and *Bam*HI digests (figure 9A, lanes 5 and 6) show additional fragments hybridizing to the *hpt*-probe, which probably represent border fragments containing neighbouring genomic DNA. Such border fragments were not observed when a *ble*-probe was used (figure 9B, lanes 5 and 6). This suggests that one or more copies of pUT720 have co-integrated with and are surrounded by pAN7-1 sequences. The patterns suggest that all pAN7-1 and pUT720 copies are arranged in the same orientation. Since the *Hin*dIII digests also show plasmid-sized bands (figure 9A and 9B, lanes 7), the tandem copies were probably obtained by ligation of the linearized plasmids before integration into the genome. Figure 9C (*hpt*- and *ble*-probes) shows a combination of the patterns obtained in panels A and B. As pUT720 and pAN7-1 have somewhat different sizes (6.04 and 6.55 kb, respectively) double bands appear around 6 kb.

The *Hin*dIII-linearized pAN7-1 and pUT720 plasmid molecules have co-integrated into the genome after ligation *in vivo*. Thus, co-integration is possible by linearizing both plasmids with the same restriction enzyme.

Example 10: Production of homokaryotic protoclones from primary heterokaryotic transformants.

Inocula from fertile, primary transformant U1mp10/Bb100-1 which included tissues from all three sectors observed, were grown on GeneScreenPlus (DuPont) hybridization membranes layered on top of cellophane sheets in petri dishes containing MMP agar medium. The plates were incubated at 24°C for 2 weeks. Then the membranes accommodating the colonies were removed, washed in 0.6 M sucrose and incubated upside down in a petri dish containing 10 mL of 0.6 M sucrose plus Novozyme 234 (10 $\mu g.mL^{-1}$) at 24°C for 1 hour to release protoplasts. Protoplasts were purified from the supernatant by sequential centrifugations with 0.6 M sucrose, resuspended in 5 mL of 0.6 M sucrose plus 1% Low Melting Point agarose (37°C) and plated onto CMPS (compost extract, Mycological Peptone, sucrose)-agar medium. Petri dishes were incubated at 24°C and individual colonies isolated, aided by microscopy, before touching their neighbours. Nineteen colonies were isolated with equal viability. From these colonies new inocula were tested for growth on DT80-medium (not shown) or DT80-medium containing 50 $\mu g.mL^{-1}$ hygromycin. Non-transformed U1mp10 was included as a control (colony A4).

The results in figure 10 show that different types of protoclones have been produced with different pigmenting (and different levels of pigments excreted into the medium surrounding the colonies, which is not visible on the photograph). Control A4 did not grow, nor did colony B3 (U1mp10/Bb100-1p14), which shows that by the procedure of protoplasting and dilute regeneration protoclones may be isolated which lost the nucleus containing the integrated *hpt*-sequences. Thus, colony B3 exclusively contains the non-transformed nuclear type, which makes this strain a homokaryon. Support for protoclonal segregation instead of deleterious recombination events is provided by the frequency obtained (1 homokaryon out of 19 protoclones tested). This frequency is expected if from heterokaryons as the starting material homokaryons of either nuclear type have been generated with an efficiency of 10%, which is the published efficiency. Further support was obtained by α-esterase isozyme-analysis (not shown). With similar frequency homokaryotic protoclones

were also identified by α-esterase analysis that still contained the *hpt*-gene. These protoclones can be used as mating partners with other homokaryons to introduce the donor DNA into a new heterokaryon.

By the method of protoplasting and dilute regeneration of primary U1mp10 (*hpt*)- transformants homokaryotic protoclones of two nuclear types can be isolated. One type, which lost the capability to grow on hygromycin-containing medium and the other type which still contained the *hpt*-gene.

Example 11: PCR-analysis of donor DNA selectable markers into protoplasts.

Protoplasts (3×10^7) were isolated and extensively washed in EB (electroporation buffer) as described in the general methods section. Then 30 µg of plasmid pAN7-1 was added, the mixture of protoplasts plus DNA was divided into three portions (10^7 each) and incubated on ice. To the first portion (1) of 10^7 protoplasts plus pAN7-1 (10 µg), plasmid pT3T7-luc (10 µg) was added directly. Portions 2 and 3 were subjected to electroporation according to the parameters described in the general methods section, incubated at 30°C for 30 min to allow sealing of pores generated by the electroporation procedure, and then transferred back to ice. To these two portions plasmid pT3T7-luc (10 µg to each portion of protoplasts) was also added followed by extensive washing with 0.6 M sucrose containing $MgCl_2$ (20 $\mu g.mL^{-1}$). Then DNAse I was added to portion 2 only (final concentration of 20 $\mu g.mL^{-1}$). Portions 2 and 3 were incubated for 30 min at 30°C, then put back on ice and lysed with phenol/chloroform simultaneously with portion 1. Total DNA was then extracted according to the method described and subjected to PCR for 15 or 20 cycles using digoxigenin-11-dUTP in the dNTP nucleotide mixture and the sets of primers indicated in the legend to figure 11. After electrophoresis the PCR-products were blotted onto a nylon membrane, treated with antidigoxigenine-AB, Fab-fragment, and AMPPD solution, exposed to FUJI medical X-ray film and developed. Portions 1, 2 and 3 correspond to fig. 11, lanes 1, 2 and 3, respectively.

Using PCR and the primer sets indicated in the legend to figure 11 it is possible to determine the entry of donor DNA into *A.bisporus* protoplasts by electroporation parameters described in the general methods section. Additional higher molecular weight bands only occurring in portions 2 and 3 possibly represent early processing of the donor DNA after entry.

Example 12: Northern blot of transformed fruiting body RNA

U1mp10/Bb50-1 fruitbodies were produced in jars essentially as described in the general methods section with adapted amounts of mycelial inocula, grain kernels, compost and casing soil. Details are described in the legends to figure 17. Fruitbodies were harvested at different timepoints. RNA was isolated from freeze-dried material and Northern blotting was carried out by standard procedures. The blot was hybridized to a ^{32}P-labelled *hpt*-probe.

Figure 12 shows a Northern blot analysis of RNA extracted from transgenic fruitbodies. After isolation the RNA showed considerable degradation on an ethidium bromide-stained agarose gel. Therefore, the signals on the Northern blot are weak and vague. However, the *hpt*-gene clearly appears to be expressed in transgenic fruitbodies.

Example 13: Southern blot analysis of Abade transformants derived with pHAG3-1

Abade transformants were obtained with plasmid pHAG3-1,linearized with *Kpn*I within the *A.bisporus* AbGH3 insert sequence. DNA isolation and Southern blot analysis were carried out as described in the general methods section.

A Southern blot analysis of total DNA from pHAG3-1-derived Abade transformants is shown in figure 13. Restriction enzyme *Cla*I does not have a recognition site in pHAG3-1. Digestion of non-transformed Abade DNA with *Cla*I and hybridization with the AbGH3-probe yields a 3.5 kb band, corresponding to the endogenous AbGH3 sequence plus flanking genomic sequences (Figure 13A, lane 3). In transformant C25-1.15/3 this band has shifted to a higher position, indicating that the pHAG3-1 DNA has integrated into the homologous AbGH3 sequence in C25-1 (Figure 13A, lane 11). This is not the case for transformant C10-1.15/3, which still shows the endogenous 3.5 kb band, in addition to a higher band, which corresponds to the pHAG3-1 sequences integrated ectopically (Figure 13A, lane 7). Both high-position bands in lanes 7 and 11 also hybridize to the *hpt*-probe (Figure 13B), confirming that they contain pHAG3-1 sequences. Both *Kpn*I and *Bgl*II have only one recognition site in pHAG3-1 and the former was used to linearize the plasmid before transformation. Digestion of C10-1 and C25-1 DNA with these enzymes yields 9.8 kb bands hybridizing to the *hpt*-probe (Figure 13B, lanes 8, 9, 12 and 13), plus additional bands representing border fragments containing adjacent genomic DNA. The plasmid sized bands of 9.8 kb indicate that in both C10-1 and C25-1

tandem copies of pHAG3-1 are present. After *Eco*RI digestion of C10-1 and C25-1 DNA two hybridizing bands of expected length appear with the *hpt*-probe, which indicate the intactness of the *GPD*-promoter region and the *hpt*-gene in the transformants (Figure 13B, lanes 10 and 14). Hybridization with the AbGH3 probe yields two different bands of 3.2 kb, corresponding to the endogenous AbGH3 sequence (Figure 13A, lane 6), and of 5.9 kb in the transformants (Figure 13A, lanes 10 and 14). The presence of the 5.9 kb band provides further evidence for the integration of tandem copies of pHAG3-1 in both transformants. Homologous integration has occurred with high efficiency (in 2 out of four transformants analyzed) when driven by the AbGH3-sequence. The mechanisms involved during integration (of pHAG3-1) are not yet fully understood, but both *in vivo* ligation and double strand break repair seem to be involved.

Transformation of Abade using plasmid pHAG3-1, linearized with *Kpn*I inside the region of homology with *A.bisporus* DNA, can give rise to homologous integration of the plasmid into the endogenous AbGH3 region. This phenomenon occurs with high efficiency (50%). In the cases of both homologous and ectopic integration investigated here, tandem copies of the integrated plasmid were found. Furthermore, in both instances the *Kpn*I-site was restored. Mechanisms resembling double strand break repair and *in vivo* ligation are involved.

Example 14: Southern blot analysis of total DNA from pHAG3-1 derived Abade transformants derived from pHAG3-1 and mated with U1 derived protoclones

Forced mating products were produced from strains indicated in the legend to figure 14 as described in the general methods section, in example 5 and figures 5A and 5B. The mating products were further propagated with no selective pressure on MMP agar medium (plus a sheet of cellophane) to isolate the total DNA for Southern blot analysis as described.

The luminograph of figure 14 shows the aberrant position of the AbGH3/*Cla*I-fragment of transformant C25-1/4/12 (lane 6). Lanes 12 - 15 comprise the DNA from different mating products with this transformed strain. Either nuclear type was clearly present in the combinations with U1p6 and U1p8, although the U1p8 nucleus seems to be under-represented in C25-1/U1p8. Over-exposure (not shown) also revealed weakly hybridizing material at the 3.5 kb position in lanes 14 and 15. These results demonstrate that by the forced mating procedure described, in two out of

four mating products analyzed the presence of both participating nuclei can be identified. It may be significant to note that the colony morphologies of mating products between Abade transformants and U1-derived protoclones was intermediate between the two parental strains with no tendency to form hyphal aggregates or sectors. The same type of morphology was also obtained after fusion of protoplasts from Abade transformants and from U1, followed by double selection (SD agar medium with no adenine, with hygromycin, 50 $\mu g.mL^{-1}$). Abade transformants D10-1 and C25-1 were also mated with protoclones from strain B131 (T.J. Elliot; ATCC36974) which is characterized by the frequent formation of 4-spored basidia. The combination with B131-protoclones allows the isolation of transgenic homokaryotic spores. A variety of new morphologies was observed after forced matings between B131-protoclones and Abade transformants on double selective medium and further propagation on MMP agar medium, including the formation of hyphal aggregates, which is also observed during early fruitbody formation. Fruitbody initiation has been started with these mating products.

The forced mating procedure described here can yield mating products in which the presence of either nuclear type can be demonstrated. In addition, unequal distribution or segregation of either one of the parental nuclei can be followed in time.

Example 15: Southern blot analysis of Abade transformants of pA1H or pU1H.

Plasmids pU1H and pA1H were constructed as described and linearized with *Eco*RI before electroporation of Abade protoplasts. Southern blot analysis was carried out as described in the general methods section.

A preliminary comparison with the transformation vectors described before, showed that the new pA1H and pU1H constructs, both containing *A.bisporus GPD2*-promoter sequences resulted in a 2 - 10 fold increase of the transformation efficiency of Abade protoplasts. Figure 15 shows that also the new constructs have been integrated into the recipient DNA. In the four transformants shown hybridizing bands of 2.1 kb were present, representing the *Eco*RI/*Bam*HI-fragments of the new constructs that comprise the 1.05 kb *GPD2*- plus 1.04 kb *hpt*-fragments.

In five transformants analyzed in more detail (results not shown) no homologous integration had occurred through the homologous *GPD2*-sequence (1.05 kb), indicating that additional factors specify the occurrence or lack of homologous integration, e.g. the nature and/or the length of the

homologous insert and/or the presence of a new *Eco*RI-site introduced at the 5'-end of the *GPD2*-promoter region by PCR (in order to allow cloning and later linearization before electroporation).

Figure 15 shows a Southern blot analysis of DNA from Abade transformants, obtained after transformation with plasmids pA1H or pU1H containing *A.bisporus GPD2*-promoter sequences. The new pA1H and pU1H transformation constructs allow the direct selection of hygromycin-resistant transformants from Abade with a somewhat increased efficiency. The *GPD2*-sequence containing vector does not integrate at the homologous position as efficiently as the vector that comprises the AbGH3-sequence (pHAG3-1).

Example 16: Southern blot analysis of an Abade cotransformant

Transformation of Abade was performed as described in the general methods section with a mixture of plasmids pAN7-1 and pUT720, both linearized with *Hin*dIII. Transformant D20-1.14/6 was selected by growth on hygromycin-containing medium. DNA was isolated and Southern blot analysis was performed as described in the general methods section.

Figure 16 shows a Southern blot analysis of total DNA from Abade co-transformant D20-1.14/6. Digests with *Nco*I/*Bam*HI and *Eco*RI/*Bam*HI of the DNA from D20-1.14/6 (lanes 15 and 16) show the same patterns as the plasmid controls pAN7-1 (lanes 2 and 3) and pUT720 (lanes 5 and 6) after hybridization with the *hpt*-probe (figure 16A) and the *ble*-probe (figure 16B), respectively. This indicates the presence of both *hpt*- and *ble*-sequences in D20-1. This is further demonstrated by the combination of patterns visible in figure 16C (*hpt*- and *ble*-probes). Digestion with *Hin*dIII (lane 17) yields plasmid sized bands with both probes, indicating that the *Hin*dIII-sites of the linearized plasmids were restored upon integration in the *Agaricus* genome. Digestion with *Eco*RV (lane 18), which does not cut either plasmid, yields one band hybridizing to both probes (figure 16C), suggesting co-integration of pAN7-1 and pUT720 at one genomic site.

Co-transformation of *A. bisporus* is possible. In the co-transformant studied in detail, it appears that the *Hin*dIII-sites, which were used for linearization of the plasmids prior to transformation, are restored e.g. by ligation of the plasmids before integration. Furthermore, the hybridization patterns observed are in agreement with integration of both plasmids at the same site in the genome.

Example 17: Effect of substrate composition on fruitbody formation.

Fruitbodies were produced from commercial strain U1 according to the protocol described in the general methods section. This time the experiments were performed in 500 mL jars, now with 25 grams of grain kernels that were inoculated with the amount of mycelium from one cellophane-covered MMP agar plate and, after colonization, with 50 grams of compost or hemp core tissue. Other handlings were in principle the same as described before.

Figure 17 shows the effect of substrate composition on the efficiency of small scale fruitbody formation. With this protocol for *A.bisporus* fruitbody production from strain U1, normally one fruitbody emerged and developed while primordia already present did not develop until the older fruitbody was removed (jar 1). Replacing the compost by hemp tissue resulted in the same type of fruitbody development (jar 2). However, with increasing amounts of added freeze-dried and finely ground compost (1% and 10% in jars 3 and 4, respectively) increasing numbers of primordia developed into fruitbodies. These results indicate that by mechanically releasing compounds from commercially available compost, which are present but not (easily) accessible to the growing mycelium, fruitbody initiation and/or development can be improved. This implies that providing *A.bisporus* with more or better enzyme activities suitable for the degradation of essential compost constituents (e.g. by co-transformation) may increase fruitbody yields.

Moreover, these results suggest that the production of mushroom fruitbodies can be applied for the bio-degradation of agro-waste materials (such as hemp core tissue). This system may be improved by the production of *A.bisporus* transformants that comprise suitable added genes.

Mushroom fruitbodies can be produced on alternative substrates. The system can be improved by the addition of compost-borne components that can be released mechanically (or perhaps enzymatically).

Example 18: Northern blot analysis of Abade transformants of pHAG3-1, pA1H and pU1H

Transformant C25-1.4/12 was obtained from *Kpn*I-digested plasmid pHAG3-1. Transformants E10-1.28/3, E20-1&2.28/3 and F10-1&2.28/3, were obtained from plasmids pA1H and pU1H, respectively, both digested with *Eco*RI. Total RNA was isolated from freeze-dried mycelium and Northern blot analysis was performed using standard procedures.

Total RNA was extracted and electrophoresed as described in the general methods section.

The results shown in figure 18 indicate that in *A.bisporus* transformants obtained with a pAN7-1-derived construct, two transcripts are generated which are full-length (arrows a and b, also occurring in *A.niger* strains transformed with pAN7-1, data not shown), whereas other transcripts are too short to yield full-length translation products (arrows c and d). The generation of two full-length mRNA products (a and b) may be explained by the two different termination signals, separated by about 250 bp, which are present in the *A.nidulans TRPC*-terminator sequence. Apparently, these are similarly recognized in *A.bisporus*. After long term propagation band c has disappeared for unknown reasons. Banding patterns from lanes 1 and 2 are representative for all transformants obtained with pAN7-1 or its direct derivative (pHAG3-1). On the contrary, Abade transformants containing vectors which comprise the *A.bisporus GPD2*-promoter sequence (and the modified *hpt*-gene) yield the normal transcripts also observed in *A.niger* pAN7-1 transformants.

Preliminary results indicate that with the new transformation construct a 2 - 10 -fold increase of the transformation efficiency was achieved in *A.bisporus*.

Using novel transformation vectors comprising the *A.bisporus GPD2*-promoter sequence (and the modified *hpt*-gene), the efficiency of *A.bisporus* transformation can be increased and the nature of *hpt*-specific transcripts normalized.

FIGURE LEGEND

Fig.1: Southern blot of undigested total DNA from eight 'Abade' transformants and controls. Lane 1, *Bam*HI-digested plasmid pAN7-1. Lane 2, *Kpn*I-digested plasmid pHAG3-1. Lanes 3-13, undigested total DNA extracted from untransformed 'Abade' (lane 3) and colonies A10-1, A25-1, B10-1, B25-2, C25-1, C25-2, D10-1, D10-2, D10-3, D25-1 (lanes 4-13, respectively). The blot was hybridized to a DIG-labelled *hpt*-probe.

Fig.2: Southern blot of digested total DNA from four 'Abade' transformants. Lane 1, *Bgl*II-digested plasmid pHAG3-1. Lane 2, *Bam*HI-digested plasmid pHAG3-1. Lane 11, *Bam*HI-digested plasmid pAN7-1. Lane 12, *Eco*RI-digested plasmid pAN7-1. Lanes 3-10, digested total DNA from transformants C25-1 (lanes 3&4), C25-2 (lanes 5&6), D10-1 (lanes 7&8) and D10-2 (lanes 9&10). DNA's were digested with *Bgl*II (lanes 3&5), *Bam*HI (lanes 4, 6, 7, 9) and *Eco*RI (lanes 8 & 10). The blot was hybridized to a DIG-labelled *hpt*-probe.

Fig.3: Schematic representation of the reconstruction of the wild-type *E.coli hpt* gene.

Fig.4A: Southern blot analysis of *A.bisporus* U1mp10-derived primary hygromycin B resistant transformants using a DIG-labelled *hpt* probe. Lanes 3-11 contain undigested total DNA and lanes 12-20 contain *Eco*RV-digested DNA from non-transformed control U1mp10 (lanes 3, 12) and transformants Bd25-1 (lanes 4, 13), Bb50-1 (lanes 5, 14), Bb50-2 (lanes 6, 15), Bb50-3 (lanes 7, 16), Bb100-1 (lanes 8, 17), Bb100-2 (lanes 9, 18), Bb100-3 (lanes 10, 19), Bb100-4 (lanes 11, 20). Lanes 1, 22: pAN7-1 digested with *Bam*HI, lanes 2, 21: size marker, phage Lambda DNA digested with *Eco*RI and *Hin*dIII.

Fig. 4B: Southern blot analysis of *A.bisporus* U1mp10-derived hygromycin B resistant sectoring transformants using a DIG-labelled *hpt* probe. Total DNAs were digested with *Eco*RI and *Bam*HI. Symbols, a,b,c: inocula taken from sectors that developed at the centre,

halfway or at the edge of the colony, respectively; p: plasmid pAN7-1 digested with *Eco*RI and *Bam*HI.

Fig. 4C: Strain-specific fingerprints of *Bam*HI+*Eco*RI double-digested total DNA from U1mp10-derived hygromycin B resistant transformants, probed with a DIG labelled *hpt* probe. Lane 1: pAN7-1 cut with *Bam*HI+*Eco*RI, lane 2: size-marker, phage Lambda DNA double-digested with *Eco*RI+*Hin*dIII, lanes 3-10: *Bam*HI+*Eco*RI double-digested DNAs from control non-transformed U1mp10 and transformants Bd25-1, Bb50-1, Bb50-2, Bb50-3, Bb100-1, Bb100-2, respectively.

Fig.5A,B: Dominant selection of a *A.bisporus* heterokaryon containing an *ade/hyg*^{R} (from C25-1) and a U1-derived nucleus (from protoclone U1p8). A: SD0-medium (no hygromycin B), with (I) or without (II) adenine. B: SD50-medium (50 µg.mL^{-1} hygromycin B), with (I) or without (II) adenine. Strain U1p8 shows clear growth on SD0-medium, but unexpectedly thin, hardly visible growth in the presence of adenine. BII (double selective medium) shows continued growth of mated colonies (duplicate inocula in the middle), exclusively, whereas parental strains do not.

Fig. 6: PCR-analysis of Abade hygromycin-resistant transformants. Lanes 1, 10: phage Lambda digested with *Eco*RI and *Hin*dIII; lanes 2 - 5: template DNA isolated from non-transformed Abade control, transformants C25-1, D10-1 and non-transformed U1, exposed to *A.bisporus GPD2* promoter-specific primers (AbGPD1, sequence id 7, AbGPD2c, sequence id 9); lanes 6 - 9: template DNA from non-transformed Abade control, transformants C25-1, D10-1 and pAN7-1, respectively, exposed to *hpt*-specific primers (PR-HPT1, encompassing ATG at position 1:
5'-ATG.AAA.AAG.CCT.GAA.CTC.ACC.GCG.ACG.TCT-3',
sequence id 10 and PR-HPT2c [complement], encompassing TAG around position 1050:
3'-GGG.TCG.TGA.GCA.GGC.TCC.CGT.TTC.CTT.ATC-5',
sequence id 11)

Fig. 7: Southern blot analysis of fruitbodies derived from U1mp10 primary transformants, hybridized to a DIG-labelled *hpt*-probe.

All DNAs were digested with *Bam*HI plus *Eco*RI. Lane 1: pAN7-1; lane 2: U1; lanes 3 - 8: transformants U1mp10/Bb50-1 (*var.* Miranda), U1mp10/Bb50-3 (*var.* Paula), U1mp10/Bb100-1 (*var.* Nicole), unstable transformant U1mp10/Bb100-2 (*var.* Nonna), U1mp10/Bb100-3 (*var.* Nadine), U1mp10/Bb100-4 (*var.* Febeline), respectively. kb, kilobase pairs.

Fig. 8A, B, C:

Southern blot analysis of total DNA from matings between primary Abade transformants and U1-derived protoclones, hybridized to a DIG-labelled *hpt*-probe. All DNAs were digested with *Bam*HI plus *Eco*RI. 8A, 8B, 8C: generation 1, 2 and 3, respectively. Lanes 1 - 18: D10-1, C25-1, U1p6, U1p6/D10-1, U1p6/C25-1, U1p8, U1p8/D10-1, U1p8/C25-1, U1p12, U1p12/D10-1, U1p12/C25-1, U1p15, U1p15/D10-1, U1p15/C25-1, U1p16, U1p16/D10-1, U1p16/C25-1, Abade, respectively and lane 19: pAN7-1. kb, kilobase pairs.

Fig. 9A, B, C:

Southern blot analysis of total DNA from an Abade-derived *hpt/ble*-cotransformant D20-1, hybridized to DIG-labelled probes: A, *hpt*-probe; B, *ble*-probe and C, mixed *hpt/ble*-probe. Lane 1: pAN7-1 digested with *Eco*RI plus *Bam*HI (2.3 kb band hardly visible); lanes 2 and 3: pUT720 digested with *Eco*RI and *Eco*RI plus *Bam*HI, respectively; lane 4: phage Lambda (digested with *Eco*RI plus *Hin*dIII, not hybridizing); lanes 5, 6 and 7: total DNA from D20-1 digested with *Nco*I, *Bam*HI or *Hin*dIII, respectively. Non-transformed Abade control did not hybridize (not shown). kb: kilobase pairs.

Fig. 10: Production of homokaryotic protoclones from primary heterokaryotic transformants. Hygromycin-resistance was tested of U1mp10/Bb100-1 (*var.* Nicole)-derived protoclones grown on DT80 agar medium containing 50 $\mu g.mL^{-1}$ hygromycin. A4: non-transformed U1mp10 control, B3: negative protoclone which has lost the hygromycin-resistance (growth without hygromycin was same as others, not shown).

Fig. 11: PCR-analysis of donor DNA *hpt*- and *LUC*-markers after re-extraction from electroporated U1 protoplasts. Lane 1: donor DNA mixture containing plasmids pAN7-1 (*hpt*) and pT3T7-luc (*LUC*); lane 2: re-extracted DNA after extensive washing plus DNAse I-treatment of protoplasts; lane 3: re-extracted DNA after extensive washing of protoplasts, only. DNA samples serving as PCR-templates were exposed simultaneously to *hpt*-specific primers PR-HPT1 (encompassing ATG at position 1: sequence id 10) and PR-HPT2c (complement, encompassing TAG around position 1050: sequence id 11) and *LUC*-specific primers PR-LUC-1 (encompassing ATG at position 304: sequence id 12) and PR-LUC-2c (complement, encompassing position 1896: sequence id 13). Panel A, B: 15, 20 PCR-cycles, respectively.

Fig. 12: Northern blot analysis of RNA extracted from fruitbodies produced from U1mp10-derived primary transformants. Lane 1: RNA from mycelium of control Abade transformant D10-2; lane 2: RNA from non-transformed U1-fruitbodies; lane 3: -; lane 4: RNA from U1mp10/Bb50-1 fruitbodies and lane 5: same strain harvested 4 days later. The arrow indicates the position of the major transcript detected in *A.niger* (pAN7-1)-transformants, corresponding to about 1450 nucleotides.

Fig. 13A, B:
Southern blot analysis of total DNA from pHAG3-1-derived Abade transformants, using DIG-labelled AbGH3- (A) or *hpt*-probes (B). Lane 1: mixture of pHAG3-1 digested with *Bgl*II or *Bam*HI; lane 2: mixture of non-digested and *Hin*dIII-digested phage Lambda DNA (not hybridizing); lanes 3 - 6: Abade non-transformed control DNA digested with *Cla*I, *Kpn*I, *Bgl*II or *Eco*RI, respectively; lanes 7 - 10: DNA from Abade transformant C10-1.15/3 and lanes 11 - 14: DNA from Abade transformant C25-1.15/3, also digested with *Cla*I, *Kpn*I, *Bgl*II or *Eco*RI, respectively. kb: kilobase pairs.

Fig. 14: Southern blot analysis of total DNA from pHAG3-1-derived Abade transformant C25-1.4/12 (showing homologous integration through the AbGH3-sequence), mated to U1-derived protoclones and

hybridized to a DIG-labelled AbGH3-probe. All *A.bisporus* genomic DNAs from controls and mating products were digested with *Cla*I. Lanes 1 and 2: pHAG3-1 digested with *Bgl*II or *Hin*dIII, respectively; lane 3: phage Lambda DNA digested with *Hin*dIII plus *Eco*RI (not hybridizing); lane 4 and 5: non-transformed Abade and U1 control DNAs; lane 6: DNA from Abade transformant C25-1.4/12; lanes 7 - 11: DNA from U1-protoclones U1p6, U1p8, U1p12, U1p15 and U1p16, respectively; lanes 12 - 15: mating products between C25-1.4/12 and U1p6, U1p8, U1p12 and U1p15, respectively. kb: kilobase pairs.

Fig. 15: Southern blot analysis of DNA from Abade transformants, obtained after transformation with plasmids pA1H or pU1H containing *A.bisporus GPD2*-promoter sequences from Abade or U1, respectively and the modified *hpt*-gene. Genomic DNAs were digested with *Eco*RV (odd numbered lanes) or with *Eco*RI plus *Bam*HI (even numbered lanes) and hybridized to a DIG-labelled *hpt*-probe. Lanes 1 and 2: non-transformed Abade control; lanes 3 and 4: transformant E10-1.28/3; lanes 5 and 6: transformant E20-1.28/3; lanes 7 and 8: transformant E20-2.28/3; lanes 9 and 10: transformant F10-2.28/3. kb: kilobase pairs; 2.1 kb, representing the *Eco*RI/*Bam*HI-fragments from pA1H and pU1H hybridizing to the *hpt*-probe.

Fig. 16A, B, C:
Southern blot analysis of total DNA from Abade co-transformant D20-1.14/6 (simultaneously transformed with pAN7-1 and pUT720), hybridized to DIG-labelled probes: *hpt*-probe (A), *ble*-probe (B) and mixed *hpt*-/*ble*-probes (C). Lane 1: phage Lambda digested with *Eco*RI plus *Hin*dIII (not hybridizing); lanes 2, 3, and 4: pAN7-1, digested with *Nco*I plus *Bam*HI, *Eco*RI plus *Bam*HI and with *Bam*HI, respectively; lanes 5, 6 and 7: pUT720, digested with *Nco*I plus *Bam*HI, *Eco*RI plus *Bam*HI and with *Bam*HI, respectively; lane 8: pABAGL-1 digested with *Eco*RI plus *Nco*I (not hybridizing); lane 9: -; lanes 10 - 14: non-transformed Abade control, digested with *Nco*I plus *Bam*HI, *Eco*RI plus *Bam*HI, *Hin*dIII, *Eco*RV or *Hin*dIII plus *Eco*RV (not hybridizing); lanes 15 - 19: D20-1.14/6, digested with *Nco*I plus *Bam*HI, *Eco*RI plus *Bam*HI, *Hin*dIII, *Eco*RV or *Hin*dIII plus *Eco*RV; kb: kilobase

pairs, sizes 6.0, 2.6 and 0.4 represent specific pUT720-fragments.

Fig. 17A, B:
Effect of substrate composition on the efficiency of small scale fruitbody formation. A: view from above, B: view from the side. Grain kernels colonized with *A.bisporus* mycelium were mixed with the substrates indicated. Jar 1: commercially available ready to use compost; jar 2, 3 and 4: hemp (*Cannabis sativa*) core tissue, remaining after removal of bast tissue, mixed with 0%, 1% and 10% (w/w) freeze-dried and finely ground compost.

Fig. 18: Northern blot analysis of total RNA from Abade hygromycin-resistant strains, transformed with pHAG3-1 (*A.nidulans GPD*-promoter and non-modified *hpt*-gene), pA1H or pU1H (containing *A.bisporus* Abade or U1 *GPD2*-promoter-sequences plus the modified *hpt*-gene described above), hybridized to a ^{32}P-dCTP-labelled *hpt*-probe. Lane 1: Abade; lanes 2 and 3: pHAG3-1 transformant C25-1.4/12, RNA from lane 2 and 3 isolated after 10 generations and after 4 generations, respectively; lane 4 - 8: pA1H- transformants E10-1.28/3, E20-1.28/3, E20-2.28/3 and pU1H-transformants F10-1.28/3, F10-2.28/3, respectively. Arrows indicate the positions of major transcripts.
Approximate sizes: a, b, c and d, 1700 , 1450, 900, 400 nucleotides, respectively, as deduced from a co-electrophoresed Gibco BRL 0.17 - 1.77 kb size marker.

Fig 19A, B, C:
Restriction maps of plasmids pHAG3-1 (A), pAnHB (B) and pAnHL (C). Abbreviations: Bm, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; ERV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; Nh, *Nhe*I; No, *Not*I; X, *Xba*I; *ble*, phleomycin-resistance gene; *hpt*, hygromycin-phosphotransferase gene; *LUC*, *Photinus pyralis* (firefly) luciferase gene; ATG, start codons; P_{GPD}, T_{TRPC}, *A.nidulans GPD*-promoter- and *TRPC*-terminator-sequences, respectively; *ssa*, excretion signal sequence of the *Trichoderma reesei cellobiohydrolase I*-gene. Essential *ble*-, *hpt*- and *LUC*-coding

sequences in grey. Numbers refer to approximate positions (bp). Individual drawings and DNA segments not to the same scale.

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(B) CLONE: Nco-HPT5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GACATCACCA TGGCTGAACT C 21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: AnTRPC3C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCGCTCGAGT GGAGATGTGG A 21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: HPT-E2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGTTCAGCGA GAGCCTGACC 20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: HPT-NTC

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CATAGCCTCC GCGACCGGCT 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EN-LUC-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGGAATTCCA TGCCATGGAA GACGCCAAAA ACATA 35

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: T7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TAATACGACT CACTATAGGG 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: AbGPD1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGAATTCGTT GTCATCACCG CTCCTGGGAG 30

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: AbGPD3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAAGAAGAAT TCAGAGGTCC GCAAGT 26

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: AbGPD2c

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GCTTATCGCC ATGGTTTGTC TCTC 24

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: PR-HPT1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 10:

ATGAAAAAGC CTGAACTCAC CGCGACGTCT 30

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: PR-HPT2c

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 11:

GGGTCGTGAG CAGGCTCCCG TTTCCTTATC 30

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: PR-LUC-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 12:

ATGGAAGACG CCAAAAACAT AAAGAAAGGC 30

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: PR-LUC-2c

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 13:

CACCGGGGCC GACTTAACCT TAGCTATAAC 30

CLAIMS

1. A method for obtaining a selectable stable transformant of a homobasidiomycete capable of expressing integrated donor DNA comprising at least a dominant selectable marker at a detectable level, wherein said host is optionally non-auxotrophic and can be transformed without co-transformation with said dominant selectable marker and is transformed with said donor DNA.

2. A method according to claim 1, wherein the host to be transformed exhibits delayed differentiation in comparison either to non-protocloned homobasidiomycete material and/or the wild type strain U1 as obtainable from ATCC, said delayed differentiation being macroscopically visible in the form of amended morphology due to a change in the number and/or height of aerial hyphae, preferably by the absence of aerial hyphae and/or a diminished hyphae aggregate formation preferably by the absence of hyphae aggregates.

3. A method according to claim 1 or 2 wherein the host to be transformed belongs to the strain 'Abade'.

4. A method according to any of the preceding claims, wherein the host to be transformed is obtained by subjecting homobasidiomycete material to protocloning followed by selection of resulting homobasidiomycete material exhibiting the desired delayed differentiation.

5. A method according to any of the preceding claims wherein the host to be transformed is obtained by subjecting homobasidiomycete material to a rejuvenation procedure followed by selection of resulting homobasidiomycete material exhibiting the desired delayed differentiation.

6. A method for obtaining a dominant selectable stable transformant of a homobasidiomycete capable of expressing integrated donor DNA at a detectable level according to claim 4 or 5, said method comprising

a) subjecting the mycelium of the host to be transformed to at least:

1) protoplast formation, followed by

2) an outgrowth phase to colonies, followed by

3) isolation of individual protoclones resulting from step 2, followed by

4) an outgrowth phase to colonies followed by

5) a selection of a protoclone resulting from step 4 on the basis of exhibiting delayed differentiation in comparison either to non-protocloned homobasidiomycete material and/or the wild type strain U1 as obtainable from ATCC, said delayed differentiation being macroscopically visible in the form of amended morphology due to a change in the number and/or height of aerial hyphae, preferably by the absence of aerial hyphae and/or a diminished hyphae aggregate formation preferably by the absence of hyphae aggregates followed by

6) optionally at least one cycle of further propagation of a selected clone including a subsequent outgrowth phase to colonies and cultivation preferably in liquid medium

7) at least one protoplast formation step from such a colony

and subsequently

b) subjecting protoplasts resulting from step 7 to transformation with donor DNA.

7. A method according to any of the preceding claims, wherein the transformation procedure occurs within 25 outgrowth phases of the detection of the delayed differentiation, preferably within 5 outgrowth phases, wherein an outgrowth phase corresponds to the growth of one clone on an agar plate of 9 cm until the plate is full

8. A method according to claim 6 or 7, wherein the protoplast to be used for transformation in step b has not been subjected to more than 5 successive outgrowth phases in step 6.

9. A method according to any of the preceding claims, wherein the host homobasidiomycete is a homokaryon.

10. A method according to any of the preceding claims, wherein the host homobasidiomycete belongs to *A.bisporus* strains U1 or 'Abade'.

11. A method according to any of the preceding claims, wherein the method of transformation is electroporation.

12. A method according to any of the preceding claims, wherein the donor DNA is linearized prior to transformation.

13. A method according to any of claims 6-12, wherein the protoplast formation step is improved by growth of mycelium in plant medium for cultivation and regeneration of plant cells, e.g. MS-medium, in particular for a host belonging to the genus *Agaricus* immediately preceding step 7 of protoplast formation.

14. A method according to any of the preceding claims, wherein the transformation is carried out using donor DNA comprising at least a vector comprising a selection marker encoding resistance to an antibiotic and/or a fungicide for which the non-transformed host is sensitive.

15. A method according to claim 14, wherein the donor DNA comprises nucleic acid capable upon expression thereof of rendering the host resistant to hygromycin as selection marker, said nucleic acid for example comprising the hpt gene of E. coli or Streptomyces or a modified derivative thereof providing increased resistance to hygromycin than the wild type *hpt* gene, preferably of *E.coli*.

16 A method according to any of the preceding claims, wherein the donor DNA further comprises a nucleotide sequence sufficiently homologous to a part of the DNA of the host to be transformed and sufficiently long to enable homologous integration to occur.

17. A method according to any of the preceding claims, wherein the donor DNA further comprises a promoter, preferably a strong promoter and optionally a terminator sequence homologous to the host to be transformed.

18. A method according to any of the preceding claims, wherein the donor DNA comprises a *GDP-2* promoter of *A.bisporus*.

19. A method according to any of the preceding claims comprising co-transformation of the donor DNA with other DNA comprising at least one further desirable sequence for transforming the host, said donor DNA and other DNA preferably being linearised and comprising termini capable of ligating to eachother, thereby linking the donor DNA to the other DNA.

20. A method according to any of the preceding claims, wherein the host material belongs to the strain Abade and is plated out on DT80 medium after regeneration and prior to selection.

21. A method according to any of claims 1-19, wherein the host material belongs to the strain U1mp10 and is plated out on B5 medium after regeneration and prior to selection.

22. A method according to any of the preceding claims, wherein the regeneration time prior to transformation comprises at least three days, preferably in CMPS medium.

23. A method for production of homokaryotic transformants from primary heterokaryotic transformants obtainable via the method of any of claims 1-22, preferably by protocloning.

24. A method for production of stable transgenic fruitbodies from primary transformed heterokaryotic material wherein at least one of the nuclei of the heterokaryotic material is transformed in a manner according to any of claims 1-22.

25. A method for production of stable transgenic fruitbodies comprising mating two compatible strains wherein at least one of the mating strains is a transformant obtainable from a method according to any of claims 1-23.

26. A method for production of stable transformants of homobasidiomycetes, comprising mating two compatible strains, wherein one of the mating strains is a transformant obtainable from a method according to any of claims 1-23 and the other mating strain comprises a further selectable marker different to the selectable marker of the transformant.

27. A method according to claim 25 or 26, wherein the transformant used in the mating is a transformant of the strain 'Abade' and said transformant comprises an adenine deficiency (as does the non transformed strain 'Abade') and resistance to hygromycin B due to the presence of donor DNA.

28. A method according to claim 27, wherein the other mating strain is not deficient for adenine and is sensitive to hygromycin B, resulting in selectability of the product of said mating on lack of adenine deficiency and resistance to hygromycin B.

29. A method according to any of claims 25-28, wherein said mating comprises naturally occurring anastomosis or artificial protoplast fusion.

30. A method of providing a genetic fingerprint specific for a transformed homobasidiomycete comprising DNA analysis of a transformant or transgenic fruitbody obtainable through a method according to any of the preceding claims.

31. A method according to claim 30, wherein a genetic fingerprint specific for heterokaryotic material resulting from a method of mating according to any of claims 25-29 can be determined distinguishing said heterokaryotic material from the homokaryotic transformant used as mating strain either by analysing for the presence of more genetic material in the heterokaryotic material than in the homokaryotic transformant and/or when the second mating strain comprises different genetic material than the transformant mating strain by analysis of the RFLP, RAPD or isozyme band pattern of the resulting heterokaryotic material.

32. Non-auxotrophic transgenic homobasidiomycete material derived from a non-auxotrophic homobasidiomycete, said transgenic material comprising stably integrated donor DNA comprising a dominant selectable marker such as a resistance to antibiotic and said transgenic material further being capable of expressing said donor DNA in an amount sufficient to ensure selectability over the corresponding non transgenic material.

33. Auxotrophic transgenic homobasidiomycete material derived from a auxotrophic homobasidiomycete, said transgenic material comprising stably integrated donor DNA comprising a dominant selectable marker such as a resistance to antibiotic and said transgenic material further being capable of expressing said donor DNA in an amount sufficient to ensure selectability over the corresponding non transgenic material.

34. A vector suitable for transforming homobasidiomycete material in particular *A.bisporus*, said vector comprising the *GPD*-2 promoter sequence

operationally connected to a selection marker encoding resistance to an antibiotic and/or a fungicide for which the host to be transformed is sensitive.

35. A vector suitable for transforming homobasidiomycete material according to claim 34 wherein said selection marker is the *hpt* gene of *E.coli* or *Streptomyces* or a modified derivative thereof providing increased resistance to hygromycin in comparison to the wild type *hpt* gene.

36. A vector according to claim 34 or 35 further comprising a sequence homologous to a part of the chromosome of the host to be transformed of sufficient homology and length for homologous integration to occur, in particular comprising the *A.bisporus* AbGH3 sequence or a portion thereof.

37. A vector according to any of claims 34-36 comprising the dominant selectable *hpt* marker, said vector obtainable by at least the following essential steps:

1) Introduction of an *Nco*I-site comprising the methionine-encoding translation initiation codon of the *hpt* gene e.g. via the PCR method using the wild-type *E.coli hpt*-gene or plasmids pHRC or pAN7-1 as templates and combinations of the following primers having seq. id 1, 2 as illustrated in the sequence listing.
2) Removal of the unique *Nco*I- and *Eco*RI-sites from the wild-type *hpt* coding region by in vitro mutagenesis e.g. via PCR using primers having sequence id 3 and 4.
3) Cloning of the fragment altered in steps 1 and 2 in a proper *E.coli* (e.g. pUC-based) vector.
4) Introduction of *Eco*RI/*Nco*I genomic fragments preferably from *A.bisporus* and optionally comprising promoter activity.
5) Introduction of *Bam*HI-*Hin*dIII genomic fragments preferably from *A.bisporus* and not containing *Eco*RI- and/or *Nco*I-restriction sites, optionally comprising terminator activity.
6) Propagation of this plasmid DNA in a proper host preferably lacking or mutated for the capacity to restrict and/or modify cloned homobasidiomycete DNA.

38. Use of a vector according to any of claims 34-37 in a method according to any of claims 1-31.

Fig-1

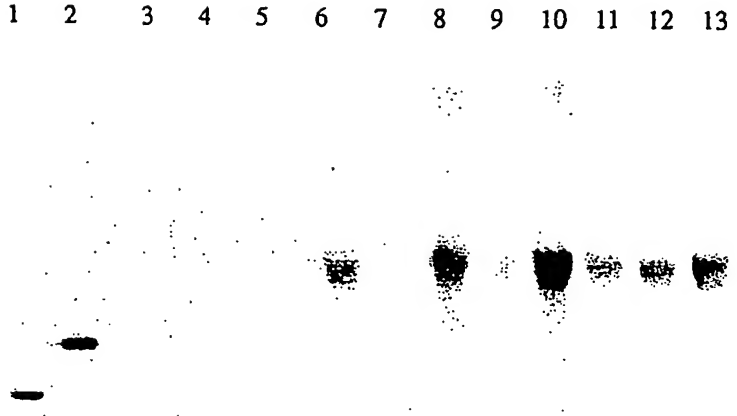


Fig-4c

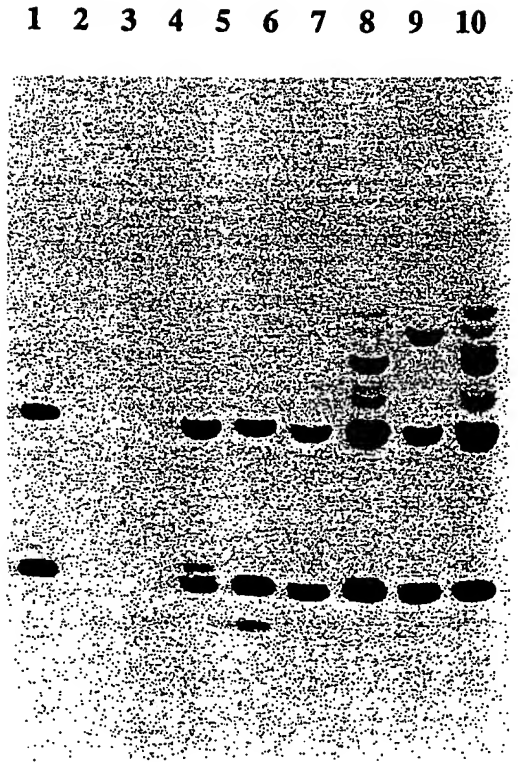


Fig-2

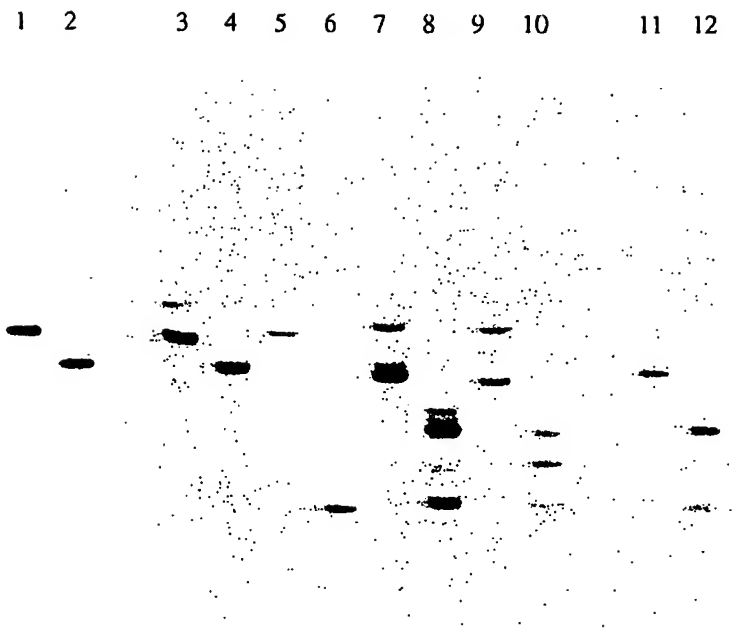


Fig - 3(1)

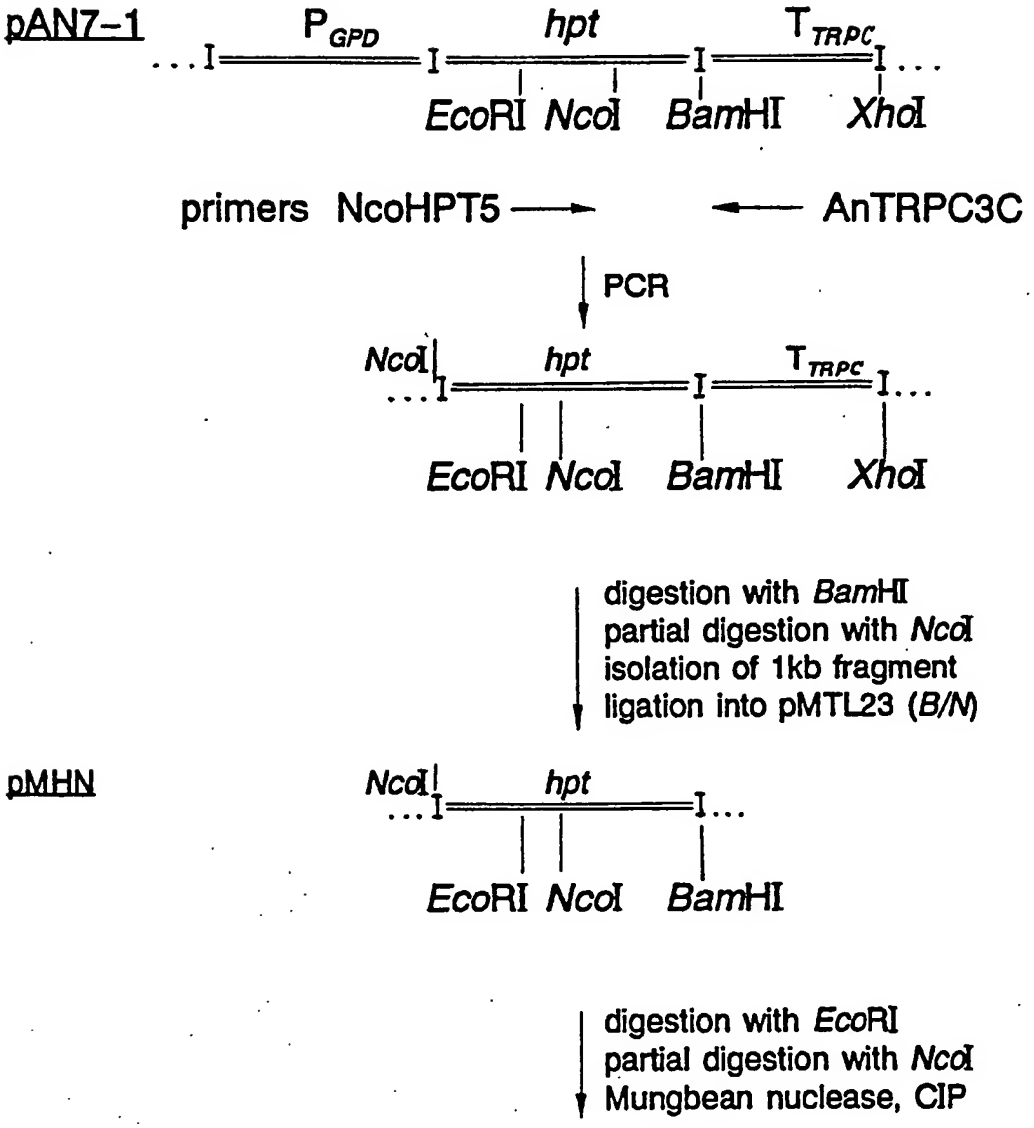
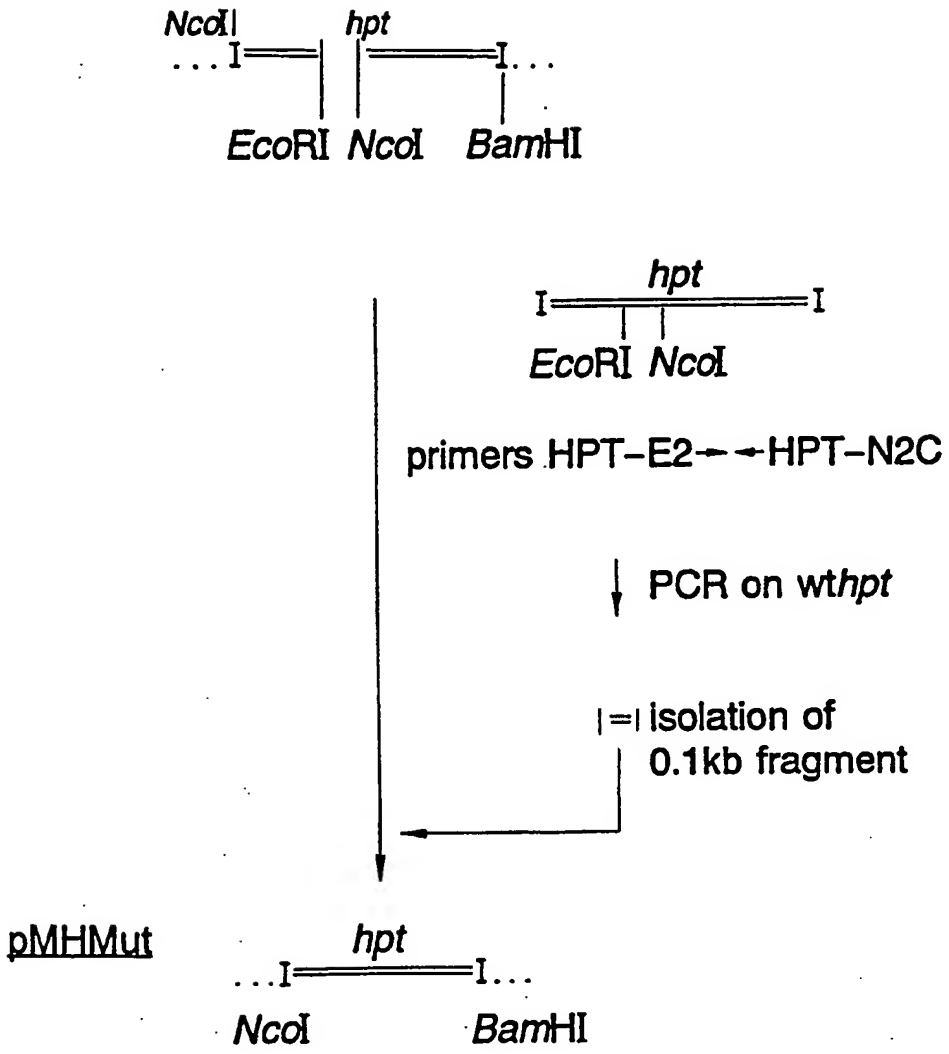

pAN7-1
P_{GPD}
hpt
T_{TRPC}
EcoRI NcoI BamHI XhoI
primers NcoHPT5 →
← AnTRPC3C
PCR
NcoI
hpt
T_{TRPC}
EcoRI NcoI BamHI XhoI
digestion with BamHI
partial digestion with NcoI
isolation of 1kb fragment
ligation into pMTL23 (B/N)
pMHN
NcoI
hpt
EcoRI NcoI BamHI
digestion with EcoRI
partial digestion with NcoI
Mungbean nuclease, CIP

Fig - 3(2)


NcoI
hpt
EcoRI NcoI BamHI
hpt
EcoRI NcoI
primers HPT-E2→←HPT-N2C
PCR on wthpt
isolation of
0.1kb fragment
pMHMut
hpt
NcoI BamHI

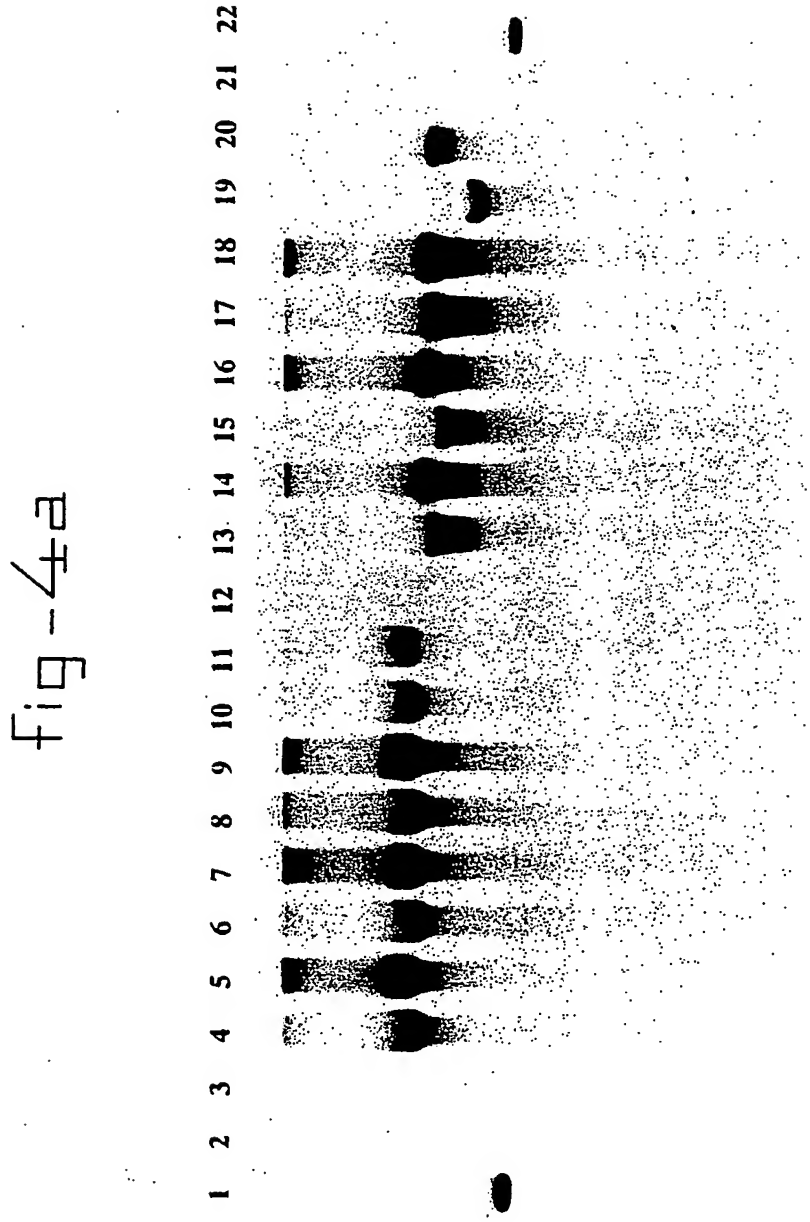


Fig-4a

fig-4b

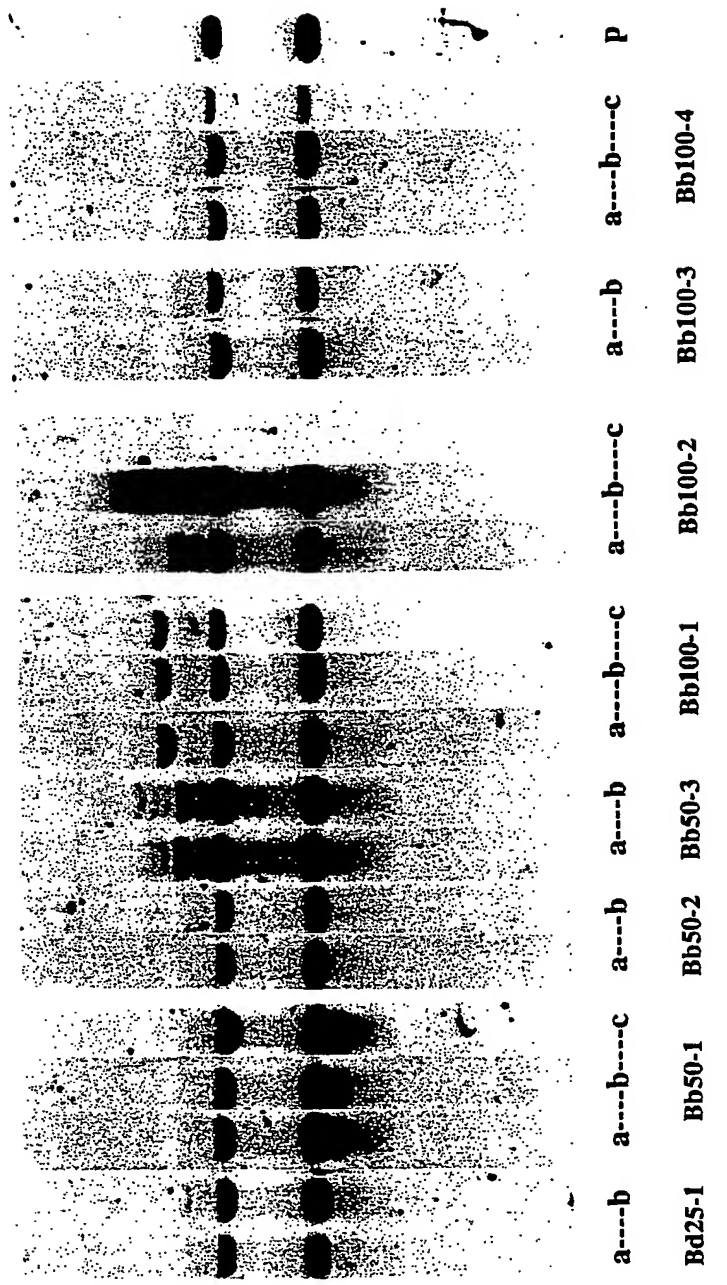
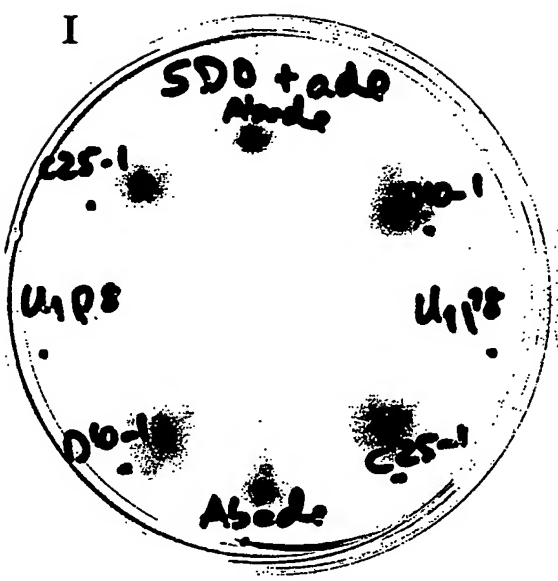

lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
sector
a---b a---b---c a---b a---b a---b a---b---c a---b---c a---b---c a---b a---b---c p
strain
Bd25-1 Bb50-1 Bb50-2 Bb50-3 Bb100-1 Bb100-2 Bb100-3 Bb100-4

Fig-5a


I
SD0 + ade
Abade
C25-1
D10-1
U1P8
U1P8
D10-1
C25-1
Abade

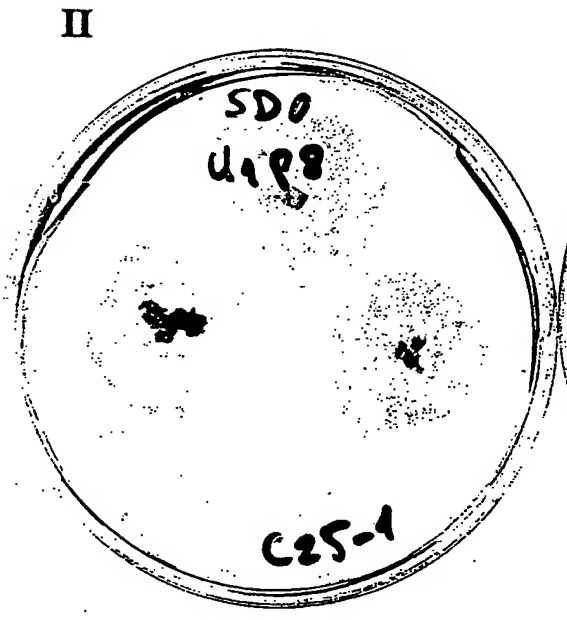
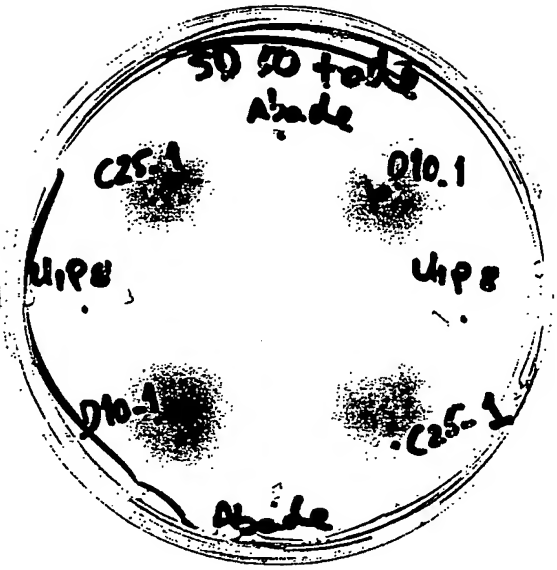

II
SD0
U1P8
C25-1

Fig-5b


SD SD + ade
Abade
C25-1
D10.1
U1P8
U1P8
D10-1
C25-1
Abade

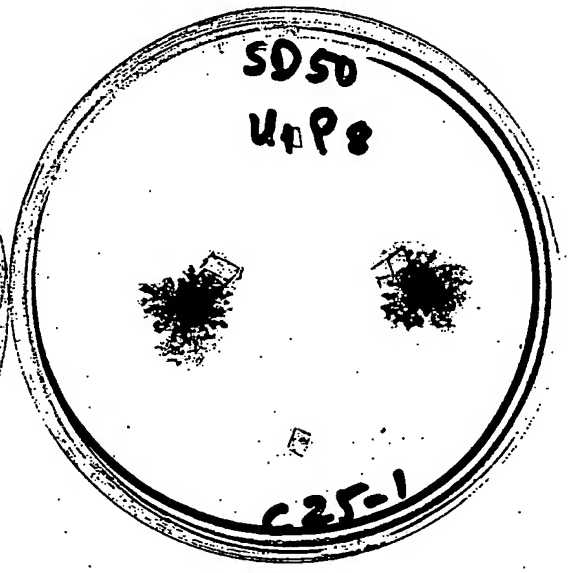

SD SD
U1P8
C25-1

Fig-6

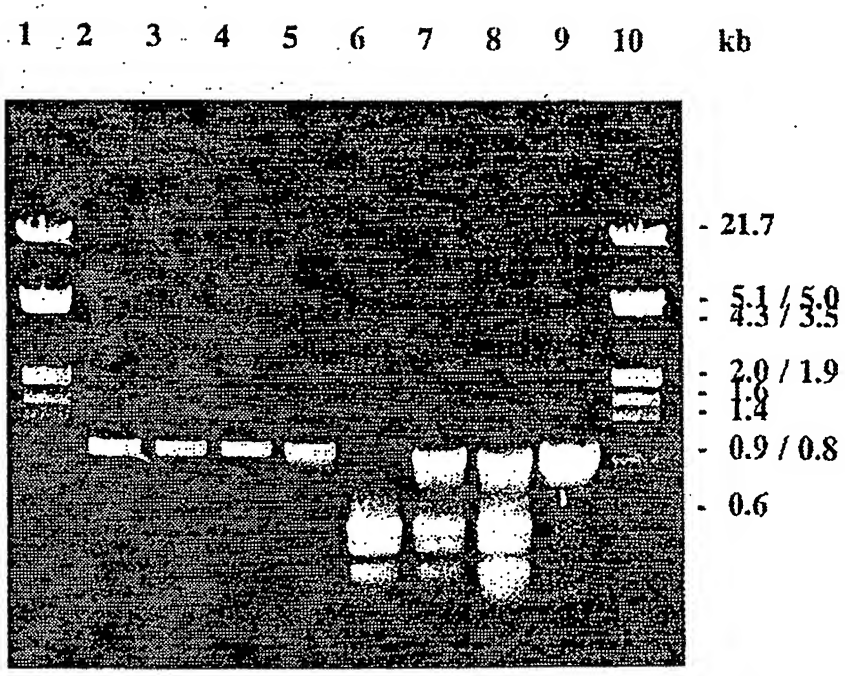
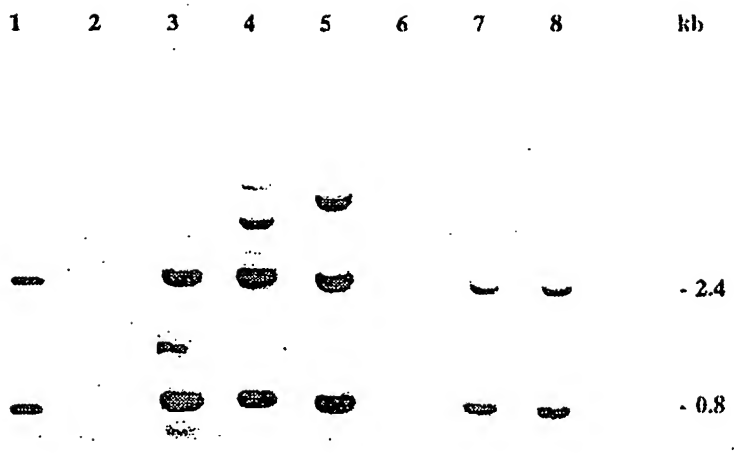

1 2 3 4 5 6 7 8 9 10 kb
- 21.7
- 5.1 / 5.0
- 4.3 / 3.5
- 2.0 / 1.9
- 1.6
- 1.4
- 0.9 / 0.8
- 0.6

Fig-7


1 2 3 4 5 6 7 8 kb
- 2.4
- 0.8

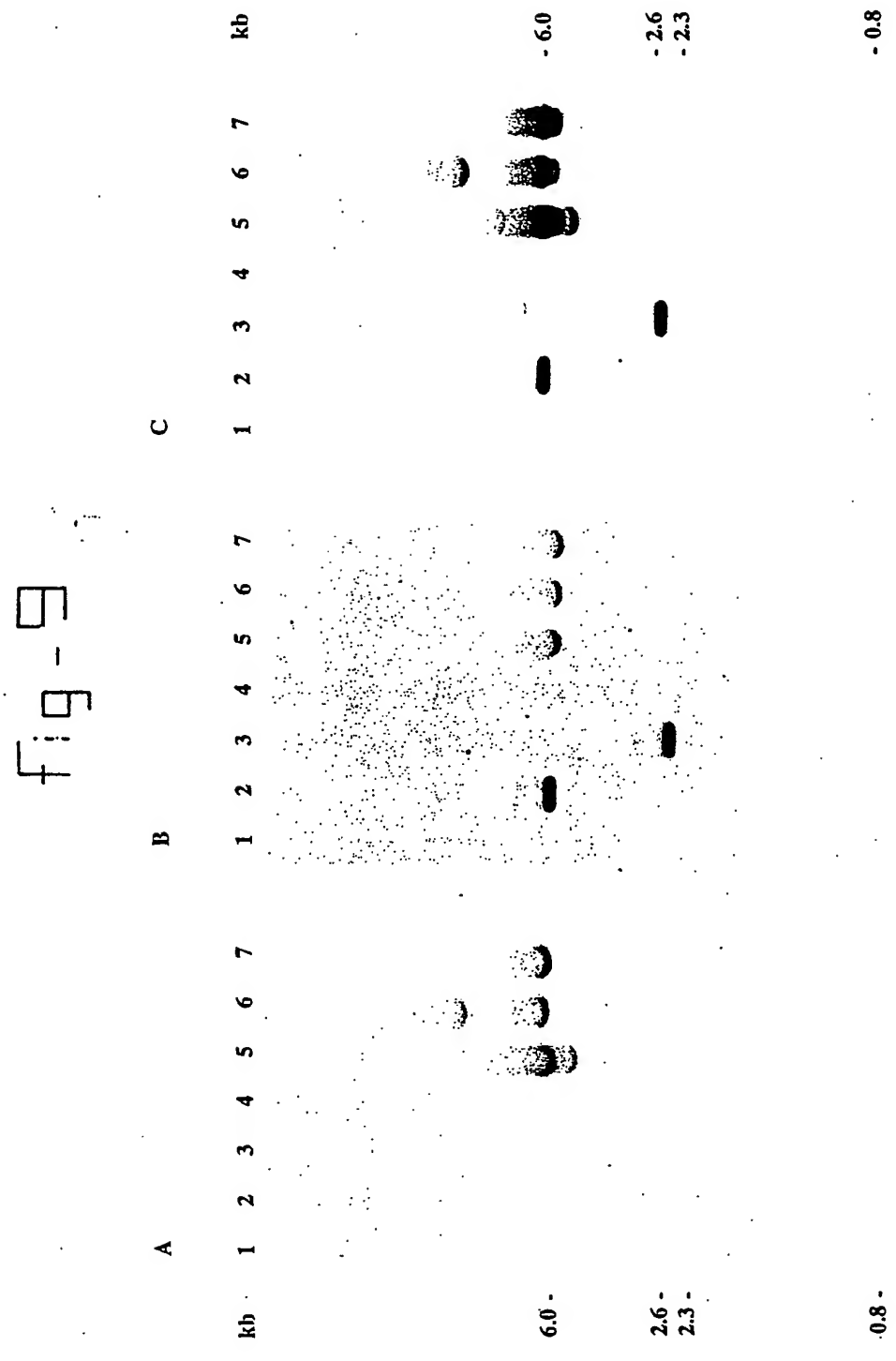


Fig-5

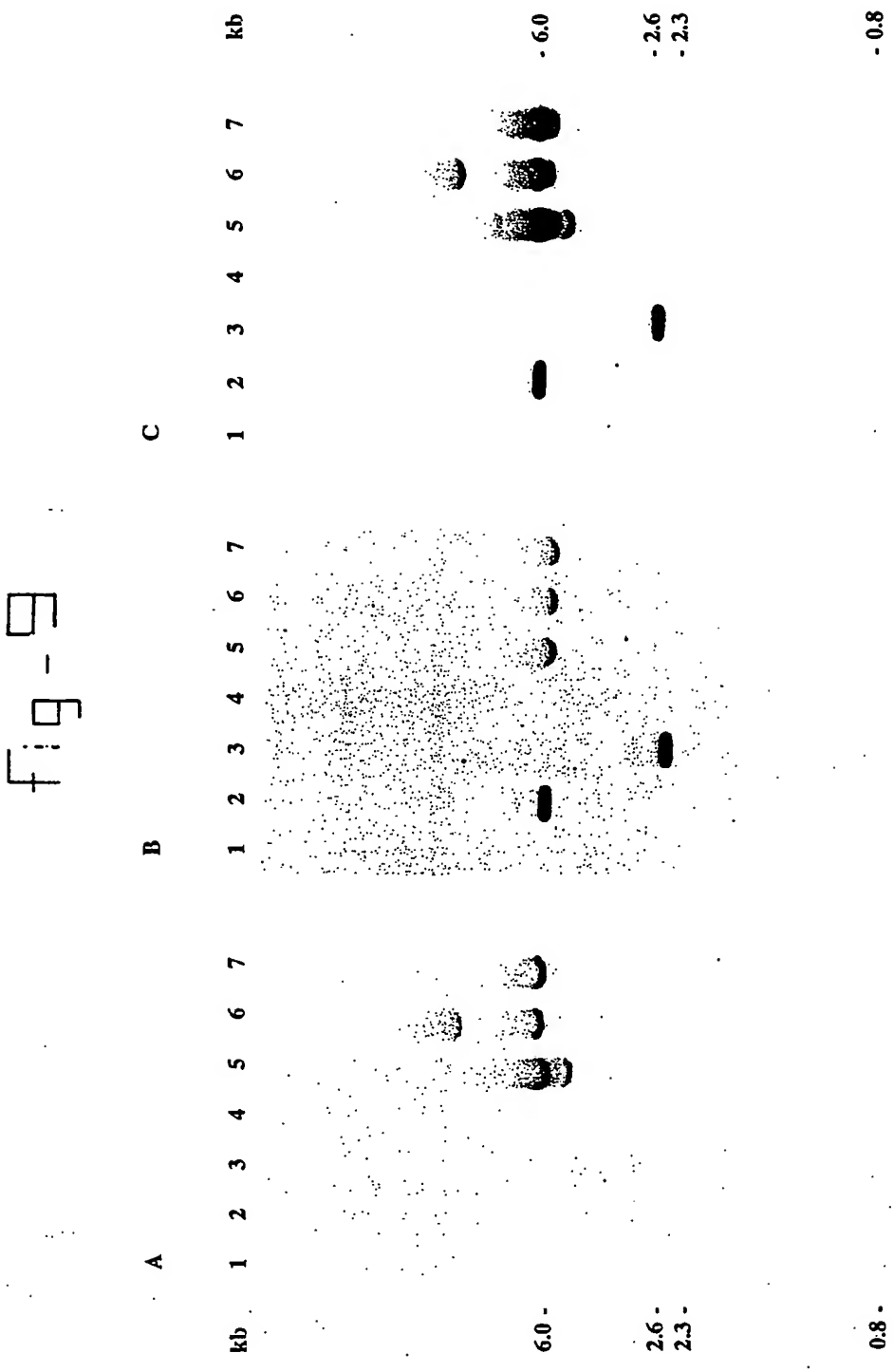


Fig-5

fig-10

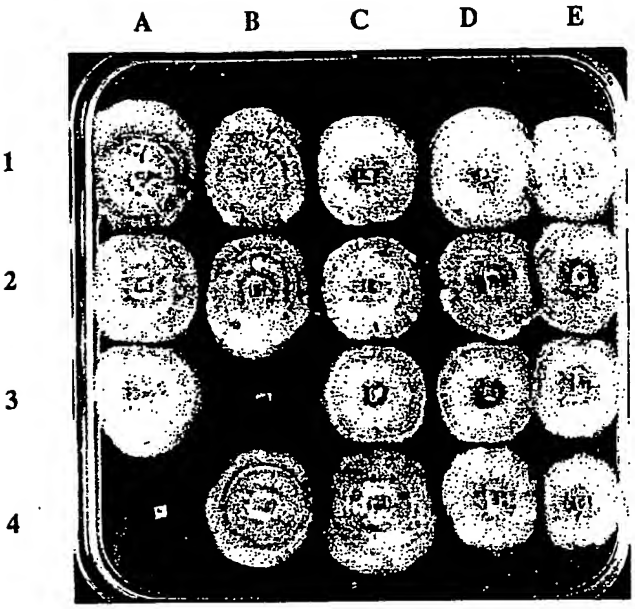


fig-11

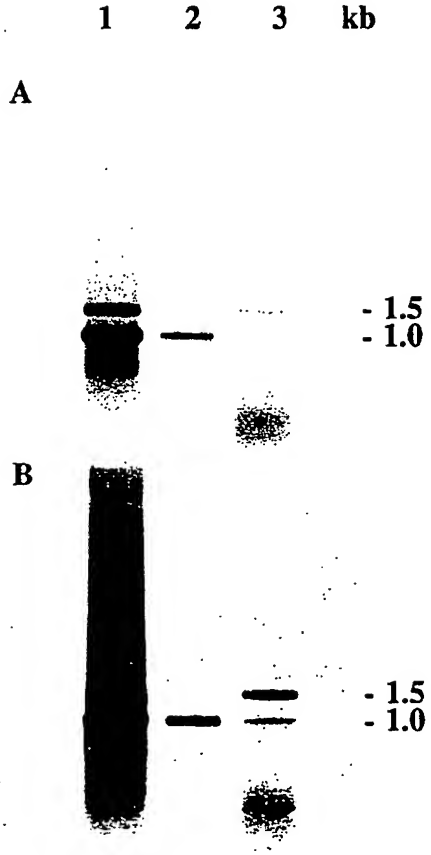


fig-12

Fig-13A

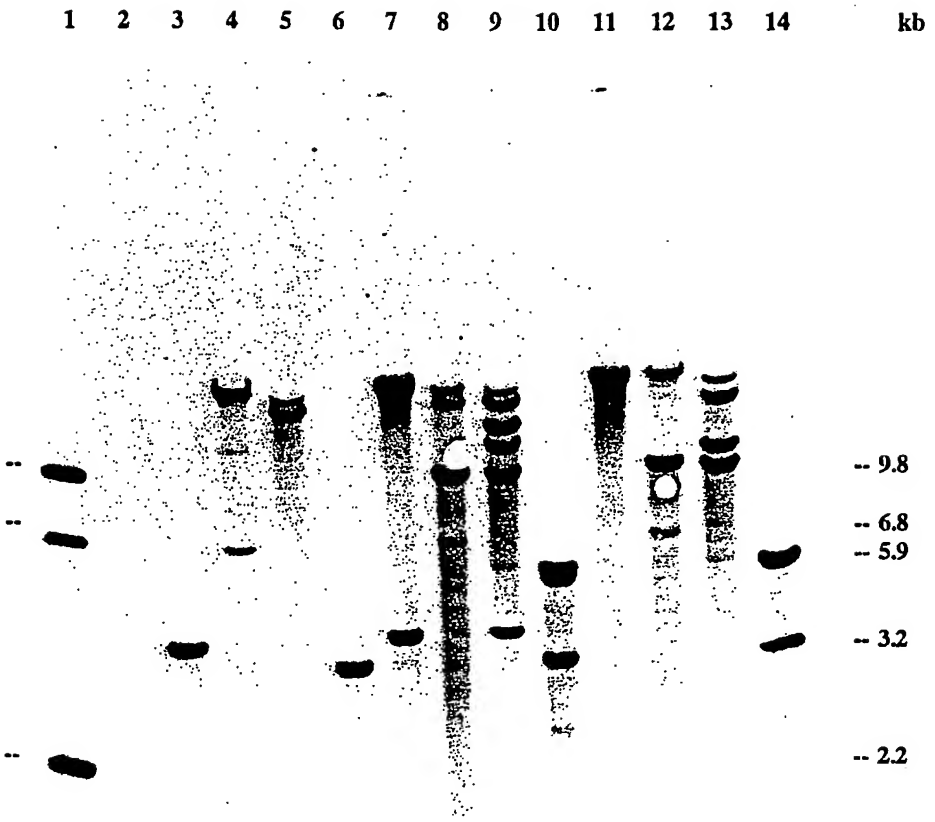


Fig-13B

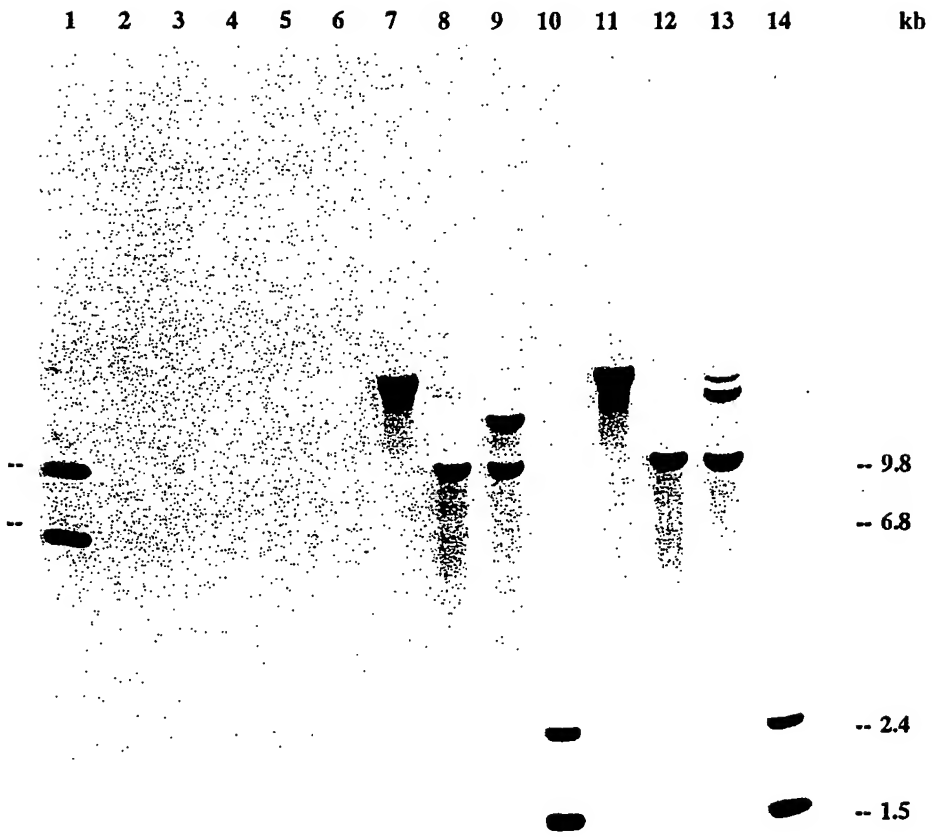


Fig-14

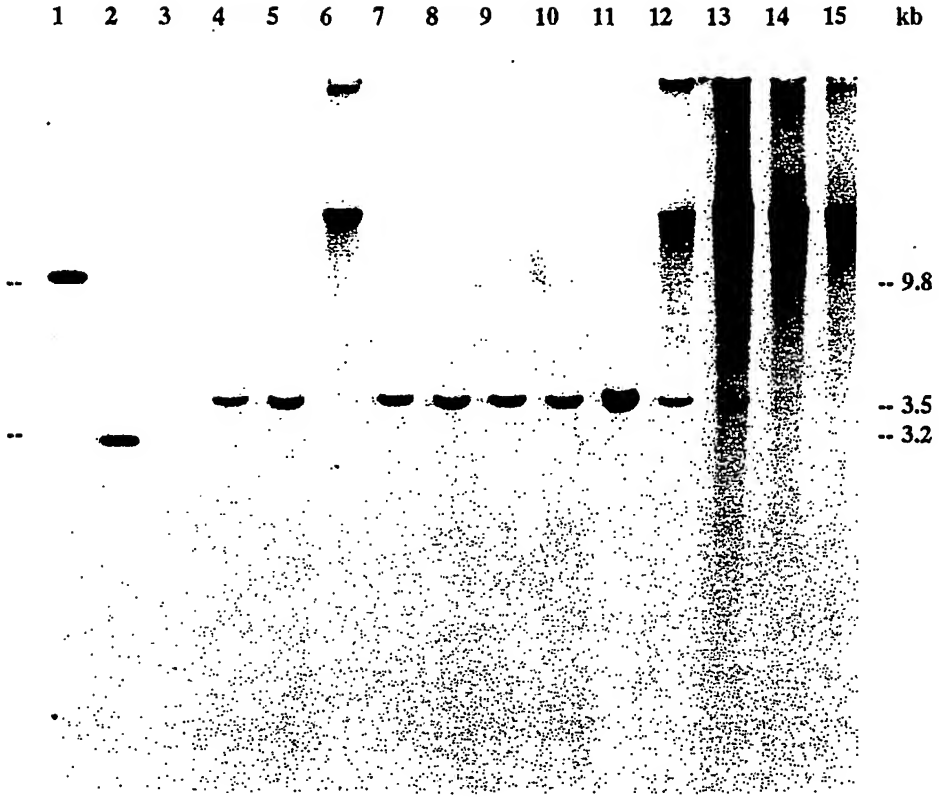


Fig-15

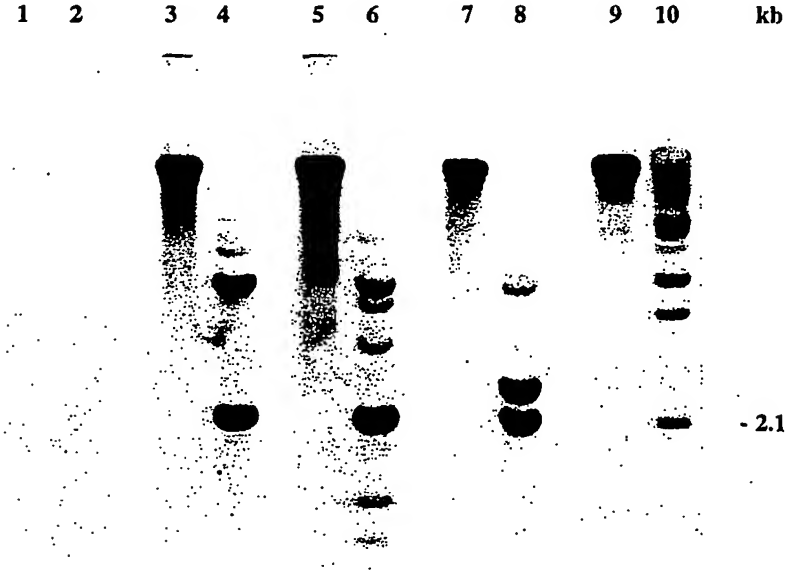


Fig -16

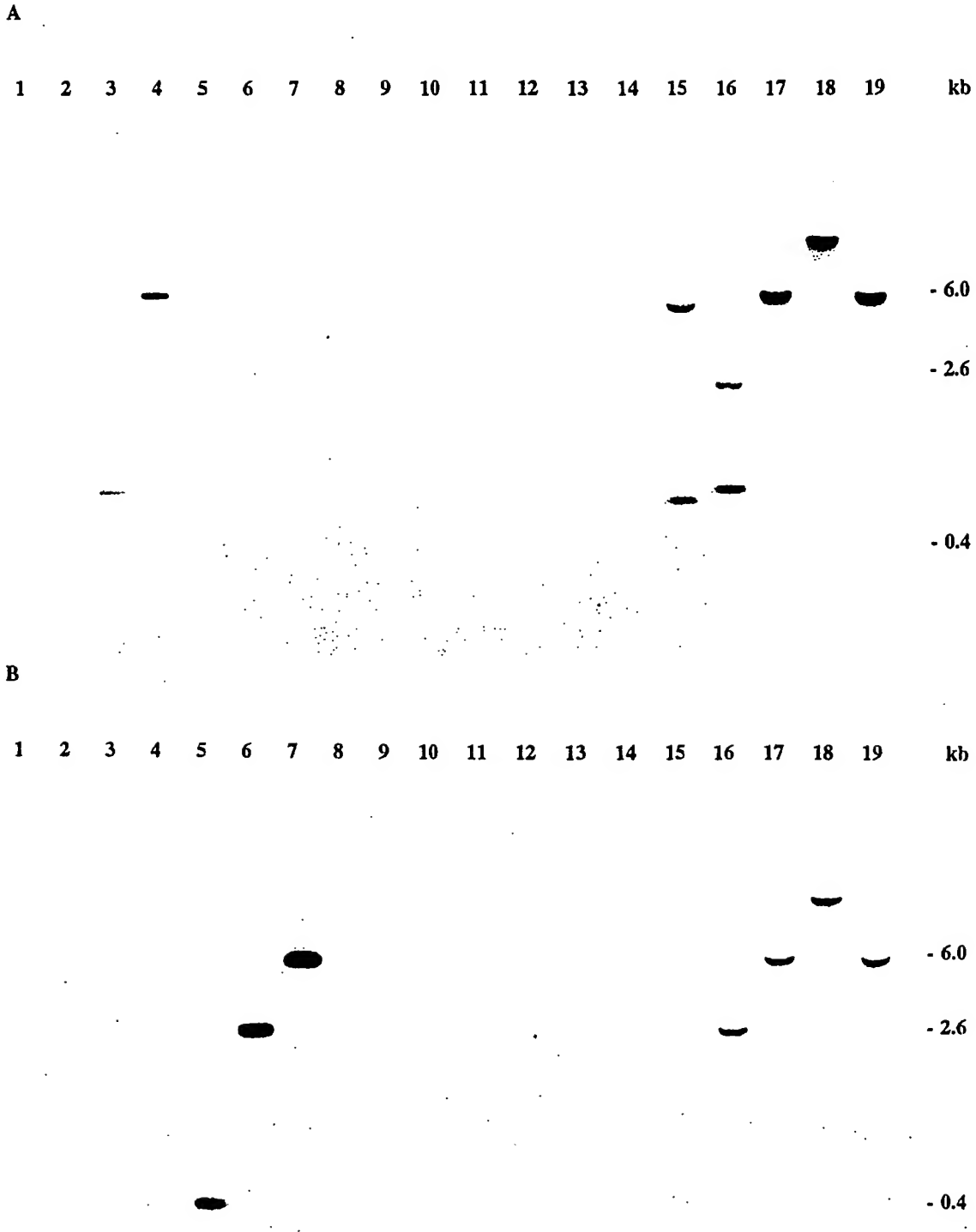

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 kb
- 6.0
- 2.6
- 0.4
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 kb
- 6.0
- 2.6
- 0.4

Fig-16

C

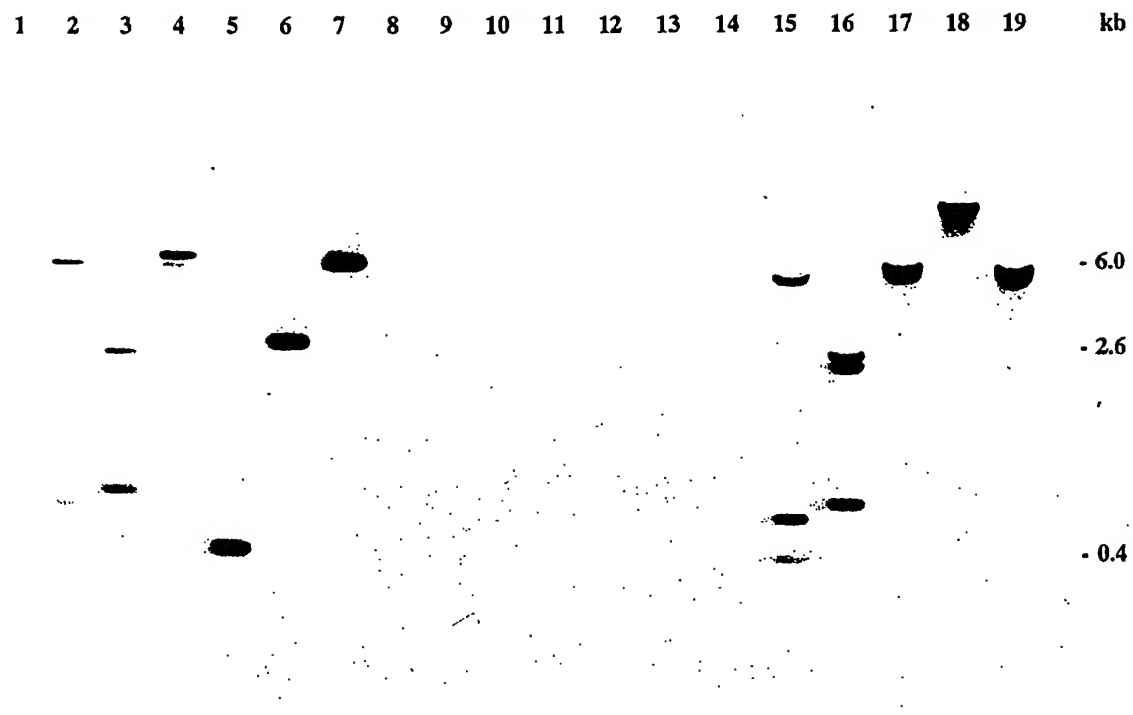


Fig -17

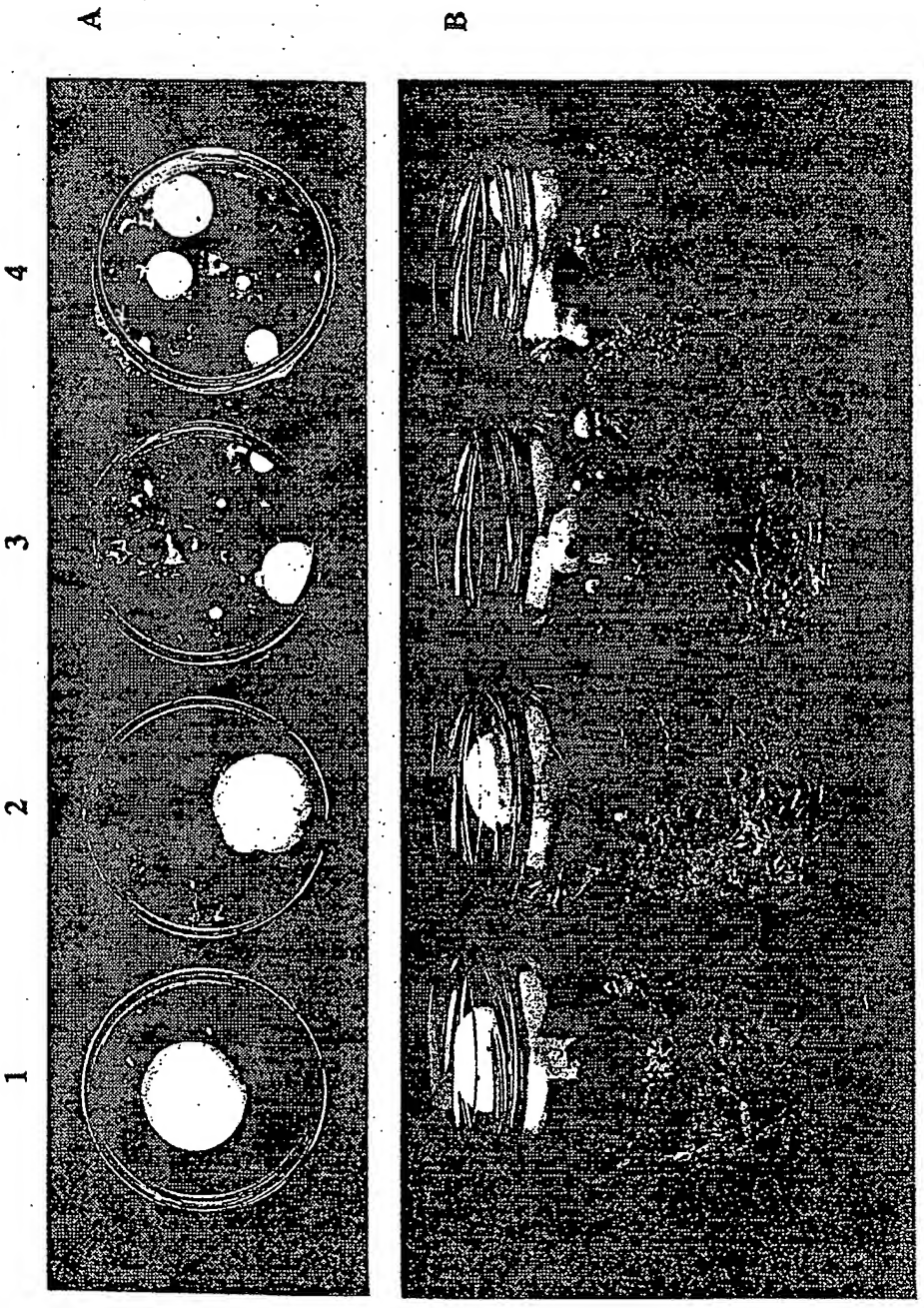


Fig-18

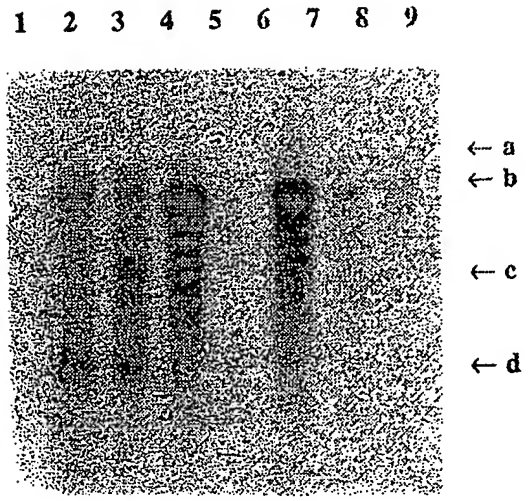
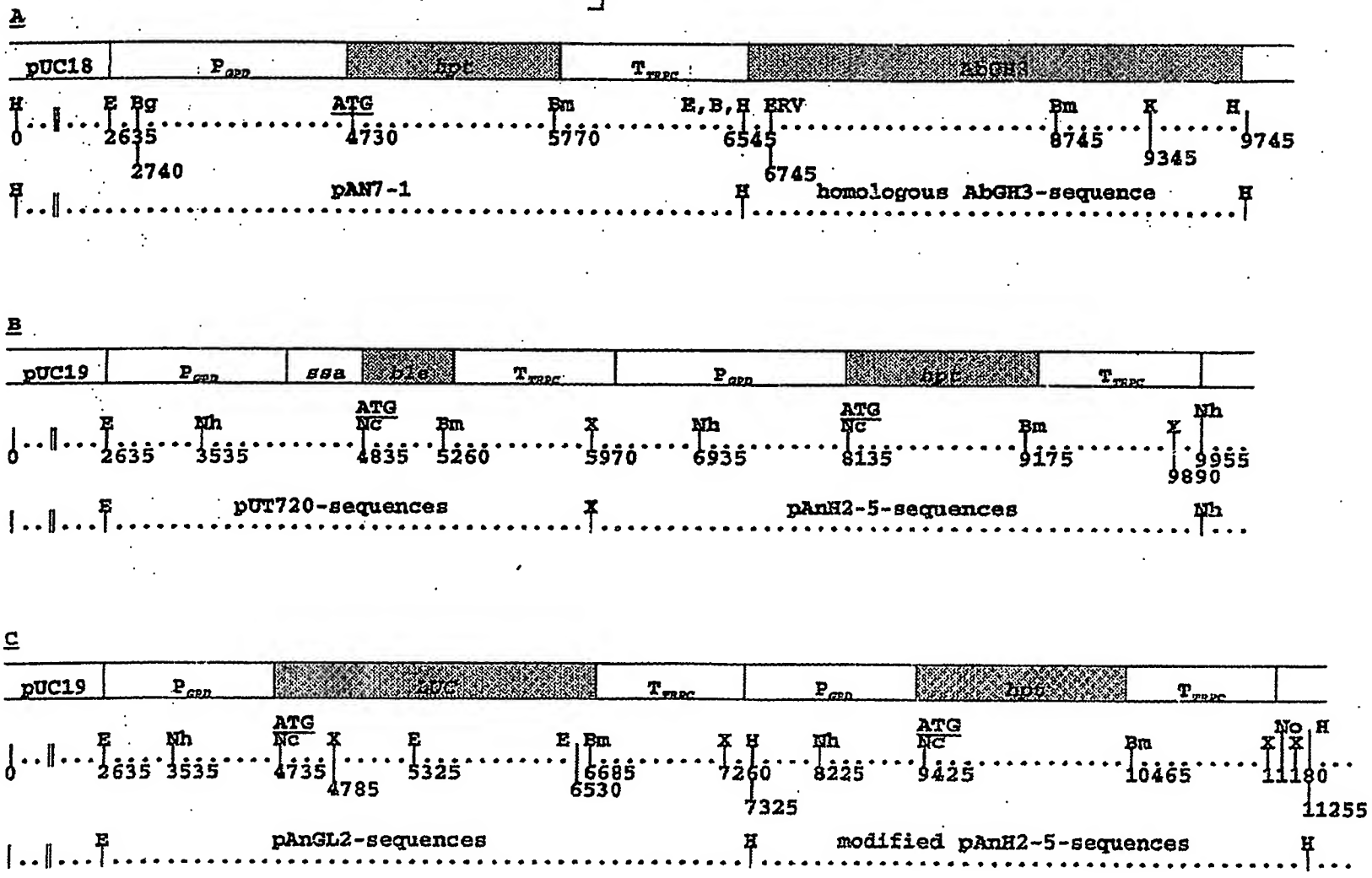


fig-19


A
pUC18
P_{GPD}
hpt
T_{TRPC}
AbGH3
H E Bg ATG Bm E,B,H ERV Bm K H
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H pAN7-1 H homologous AbGH3-sequence H
B
pUC19
P_{GPD}
sba
ble
T_{TRPC}
P_{GPD}
GFP
T_{TRPC}
E Nh ATG/NC Bm X Nh ATG/NC Bm X Nh
0 2635 3535 4835 5260 5970 6935 8135 9175 9890 9955
E pUT720-sequences X pAnH2-5-sequences Nh
C
pUC19
P_{GPD}
AUC
T_{TRPC}
P_{GPD}
AUG
T_{TRPC}
E Nh ATG/NC X E E Bm X H Nh ATG/NC Bm No X H
0 2635 3535 4735 4785 5325 6530 6685 7260 7325 8225 9425 10465 11180 11255
E pAnGL2-sequences H modified pAnH2-5-sequences H